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FACTORS AFFECTING THE RETENTION OF ENZYMES BY HUMAN ERYTHROCYTE GHOSTS

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Factors Affecting the Retention of Enzymes by Human Erythrocyte Ghosts" submitted by Gusztav Duchon in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

This investigation is a study of some factors that may influence the association of enzymes with human erythrocyte ghosts, as well as the effect of these factors on membrane integrity.

The osmolarity of the wash medium has been found to have a pronounced influence upon the final enzyme content of the erythrocyte ghosts. When ghosts prepared in 80 mOsm buffer were subsequently washed in water, some of the enzymes were liberated into the supernatant, while others were retained by the ghosts. This finding provided the basis to classify the enzymes as "loosely-bound" and "firmly-bound". "Basic activity profile" curves of the enzymes were obtained by assaying ghosts - prepared in buffers of various osmolarities - in the corresponding buffers. These curves revealed that the retention of loosely-bound enzymes was minimal between 5 - 10 and maximal at 30 mOsm buffer concentrations. The basic activity profile curves of the firmly-bound enzymes were descending as the osmolarity of the wash media was increasing. A large enhancement in enzymic activity (Cryptic activity) was found if the osmolarity of the ghost suspension was reduced, or if the ghosts were treated with Triton X-100, prior to the start of the reaction.

While the pH of the wash medium had only a slight effect on the retention of firmly-bound enzymes, it markedly affected the loosely-bound enzymes, which were preferentially retained by the erythrocyte ghosts at lower pH. The loosely-bound enzymes could be liberated from or recombined with the erythrocyte ghosts, by a simple adjustment of the pH of the suspending medium.

The presence of either Mg or Ca in the wash medium greatly affected the final hemoglobin content of the erythrocyte ghosts, as well as influenced the retention of loosely-bound enzymes. The enhancement in enzyme retention due to Mg or Ca was "latent" in nature, since it required the presence of Triton X-100 in the reaction mixture to be revealed. These ions however proved to be ineffective in influencing the retention of firmly-bound enzymes. Phase-contrast photomicrography showed that ghosts disintegrate when the osmolarity of the suspending medium is reduced below 30 mOsm. In the course of hypotonic disintegration, formation of long ribbon-like tubules or alternatively formation of vesicles was apparent. The hypotonic disintegration of ghosts could be prevented by incorporating 200 μM of either Mg or Ca ions in the wash medium.

Erythrocyte ghosts treated with Triton X-100 were found to contract to 0.13 of their original volume in the presence of ATP, but not in the presence of any of the other triphosphate nucleotides examined here.

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α-GPDH	α-glycerophosphate dehydrogenase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATP-ase	adenosine triphosphatase
CTP	cytidine triphosphate
CD	circular dichroism
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetra-acetate
GSH	reduced glutathione
GSH-P	glutathione peroxidase
GSSG	oxidised glutathione
GSSG-R	glutathione reductase
IU	international unit, μmole per min.
LDH	lactate dehydrogenase
MES	2(N-morpholine)ethanesulphonic acid, pK = 6.15
MTT	equimolar concentration of MES, TES, and TRICINE
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ORD	optical rotatory dispersion
PGK	phosphoglycerate kinase
PI	inorganic phosphate

PK	pyruvate kinase
TCA	trichloroacetic acid
TEA	2,2',2"-nitrilotriethanol
TES	N-tris(hydroxymethyl)-methyl-2-amino ethanesulphonic acid, pK = 7.50
TPHD	triosephosphate dehydrogenase
TPI	triosephosphate isomerase
TRICINE	N-Tris(hydroxymethyl)glycine, pK = 8.15
Tris	tris(hydroxymethyl)aminomethane, pK = 8.1
UTP	uridine triphosphate
TX-100	Triton X-100

I. INTRODUCTION

For many years the red cell membrane was thought to be only a permeability barrier, an envelope enclosing the hemoglobin and other cytoplasmic substances, and the control of permeability is still regarded as its prime function. The structure of the membrane however is complex, and the accumulating evidences suggest that its function is more than a simple permeability barrier. The red cell membrane has a protective and mechanical function. It was shown by Guest et al. (1), using high speed cinephotography, that the red cells are easily deformable into a cup shape on passing through small capillaries, after which they quickly recover their characteristic biconcave shape. This biconcave shape - which is unfavorable hydrodynamically - allows some swelling to occur, without an increase in surface area (2), providing protection against hemolysis in case the osmolarity of the medium decreases. Finally the membrane is the site of interaction of the cell with its surrounding plasma, and some components of the cell interior may be transiently located in the membrane, and play an integral part in its metabolism (3-7).

As in most cells, the largest constituent of the red cell is water, which can flow rapidly across the cell membrane, and a predominantly inward flow causes hemolysis. This inward flow of water can be achieved by reducing the ionic strength of the suspending medium, which results in swelling of the red cell, until a critical volume is reached, above which lysis will occur. Prior to lysis the membrane will stretch and hemoglobin will leak out all

over the surface (8), leaving the membrane intact. The term "ghost" is used in preference to membranes, for description of the delicate discoidal bodies obtained after the removal of hemoglobin from the erythrocytes. Ghosts can not be stained, but they can be viewed under phase optics, and, depending on the method of preparation, the intact ghost may closely resemble the shape of the erythrocyte. They regain under certain conditions their biconcave shape, and behave as almost perfect osmometers, being semipermeable in the sense that sodium and potassium ions exhibit a slow diffusion in both directions while water is rapidly transferred across the ghost membrane in such a direction as to secure continuous isotonicity with the external medium (9).

The isolation of the erythrocyte membrane is fundamental to the detailed study of its composition. Representative techniques for the preparation of red cell ghosts include hemolysis in distilled water as proposed by Bernstein (10), the freeze-thaw method described by Jorpes (11), the use of chemical lysins such as digitonin, and the use of 0.1% sodium chloride solution saturated with carbon dioxide (12). The most recent techniques are dependent on successive osmotic hemolysis of the erythrocyte suspension (13-16). The hemoglobin content of these different ghost preparations is as widely varied as the techniques themselves. While Bernstein obtained 30% hemoglobin as dry weight for his ghost preparation, it was reduced to 0.2% by the technique of Dodge *et al*. (14).

Modern advances in lipid chemistry have made possible accurate and fairly consistent analyses of red cell lipids. Depending upon the method of preparation of ghosts the amount of lipid can vary from 30%

to 50% of the dry weight of the membrane. Seventy percent of the lipid is phospholipid, and the remainder is cholesterol. The phospholipids have been extensively fractionated by means of chromatography on silicic acid and alumina, with mixtures of various organic solvents. A little over half of the phospholipid is composed of the choline-containing lipids, phosphatidyl choline and sphingomyelin, the bulk of the remaining being phosphatidyl serine and phosphatidyl ethanolamine. Unsaturated fatty acids make up 73% of the total lipid fatty acid composition, of which 50% is oleic acid, and 17% arachidonic acid (17-19). Cholesterol - which readily exchanges with serum cholesterol - is localized on the periphery of the biconcave disc as was shown by Murphy (20), using autoradiographic studies with tritium-labeled cholesterol. He proposed that the resulting interfacial tension in different areas of the membrane results in the discoidal shape of the erythrocyte.

Compared with the analysis of red cell lipids, the proteins of the erythrocyte ghost have been poorly characterized, which can be attributed to the insolubility of the membrane. Once the red cell membrane has been formed, the protein is apparently stable. Studies with ^{15}N glycine (21) indicate no incorporation into the proteins of the membrane.

The first attempt to characterize the proteins of the erythrocyte ghost was carried out by Jorpes (11). He extracted his ghost preparation with a mixture of ethanol and ether, and called the remaining protein "stromatin". Moskowitz and Calvin (22) extensively washed red cells with water, and then with alkali at pH 9,

and called the remaining sediment "stromin". After extracting stromin with ether, a phospholipoprotein fraction was recovered and named "elinin". It was found to be fibrous in character with a molecular length of 2500 Å and a width of 125 Å. Ultracentrifugal data indicated a particle weight of 40×10^6. They have suggested that elinin fibers might lie parallel to the cell surface.

Rega et al. (23) have isolated lipid-free protein, by extraction of ghosts with n-butanol at 0°. They found the protein to be soluble in aqueous salt media at neutral pH, to have a single electrophoretic mobility, and to have a single peak in Sephadex gel column chromatography; ultracentrifugal analysis however indicated heterogeneity of the protein. The freshly extracted protein had cation-independent ATP-ase (ATP-phosphohydrolase, EC 3.4.1.4) activity.

Azen et al. (24) solubilized ghost proteins by treating lipid-extracted membranes with aqueous urea and mercaptoethanol. They subjected the protein extract to starch-gel electrophoresis, and found they were able to obtain a large number of components. Although this treatment did not yield native protein, they felt that the procedure might be useful, in detecting genetic abnormalities of the membrane. Zail et al. (25) have tested their predictions on patients with hereditary spherocytosis, but found no significant qualitative variations in the electrophoretic patterns.

Schneiderman (26) solubilized ghosts by mixing them with an equal volume of 5% Triton X-100 in 8M urea and an equal volume of mercaptoethanol. Electrophoresis on polyacrylamide gel revealed a

number of migrating components, which were protein in nature. The slowest migrating band - which also stained positively for lipids - showed alkaline phosphatase activity.

Using hypertonic sodium chloride solution (0.2 - 1.4 M) Mitchell and Hanahan (27) achieved partial solubilization of lipids, acetylcholinesterase (acetylcholine acetylhydrolase EC 3.1.1.7), and some other proteins of the erythrocyte ghosts, without complete disruption of the underlying stromal structure.

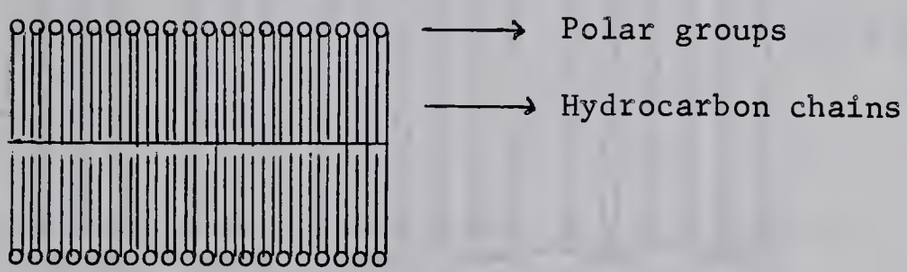
Marchesi et al. (28) have solubilized 20% of the protein associated with the erythrocyte ghost, by dialyzing the ghost preparation against ATP and 2-mercaptoethanol. The protein, which they named "spectrin", was found to polymerize in the presence of divalent cations to form coiled filaments, the ultrastructure of which was similar to the actin filaments of muscle; but it was different in physical, chemical, and antigenic properties from muscle actin derived from the same animal. Spectrin was furthermore found to be different antigenically from the serum proteins and cytoplasmic proteins released from the erythrocyte during hemolysis.

An insight into the actual structure of the membrane came initially from theoretical and physical considerations. However, the advent of the electron microscope technique has allowed a more direct visualization of the structure. The idea that lipids were important components of the membrane originated with the observation that the rate of permeation of solutes was related in a general way to their lipid solubility. This observation suggested that the permeability barrier was predominantly lipoidal. The analytical work of Gorter and Grendel (29) further

strengthened the belief in the importance of lipids in membrane structure. They found that the lipids extracted from erythrocytes occupied, in a Langmuir trough, twice the surface area of the equivalent number of red cells. Since all the lipids are located in the erythrocyte ghosts, the finding suggested a bimolecular layer of lipids in the membrane, with the polar groups on the surface, and the hydrocarbon chains making up the interior as indicated in diagram 1.

The contribution of protein to the membrane was suggested from considerations of the interfacial tension at the membrane surface. For a membrane consisting of a bimolecular lipid leaflet the tension at the

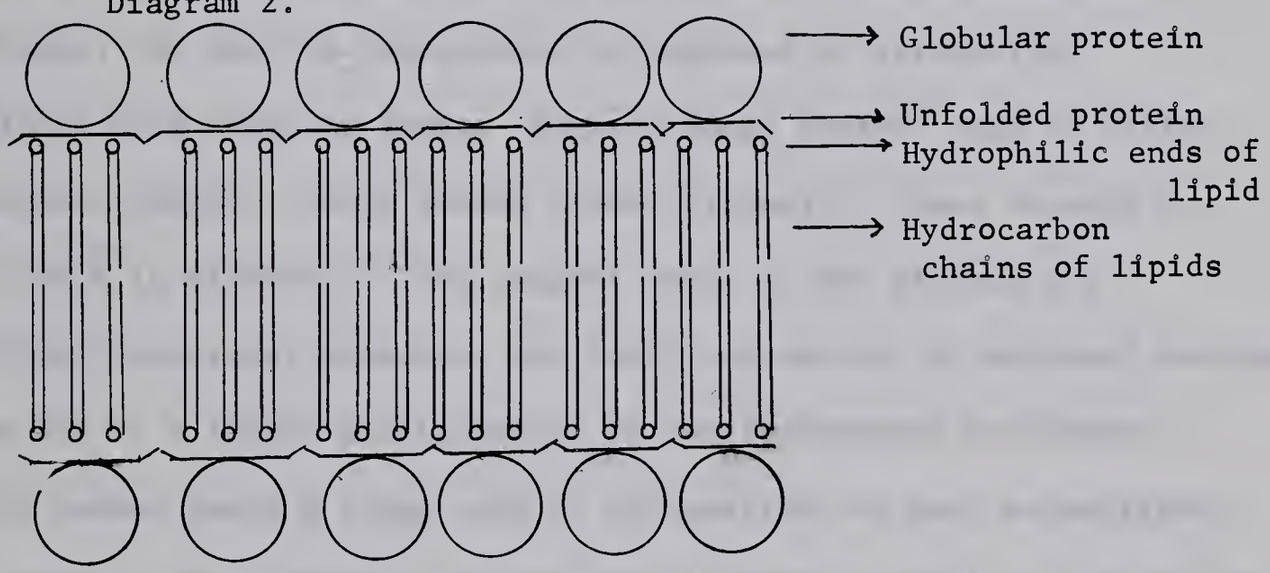
Diagram 1.



surface of the cell would have to be 10-20 dynes/cm, whereas the actual tension was found to be 0.1 dynes/cm. (30). Danielli and Harvey found a protein component to be responsible for these low tensions and subsequently showed that many other proteins had an equally high surface activity. The observation that proteins form insoluble films at interfaces, the thickness of which is 7 Å or less, led to the conclusion that the large molecules must unwrap themselves at the interfaces, destroying the secondary and tertiary structure of the molecules (30). At low surface pressure the side-chains would lie flat

on the interface. On an increase in the pressure they would be forced into a vertical position, with the nonpolar side-chains projecting into the hydrocarbon chains of the lipids, and the polar linkages oriented toward the water phase. This latter condition would create the insoluble gel state. The Danielli-Davison theory - which has dominated the thinking about the molecular organization of the membrane for over thirty years - has gradually emerged as a bimolecular lipid layer sandwiched between two protein layers as indicated in Diagram 2.

Diagram 2.



A more direct visualization of the red cell membrane is obtained with the use of the electron microscope. Electron micrography of sectioned human erythrocytes, fixed and stained with potassium permanganate, can be interpreted in a way that would support the Danielli-Davson model.

Hillier and Hoffman (31) have treated red cell ghosts with phosphotungstic acid, shadowed them with metal, and taken face-on electron micrographs. Their pictures show a pebbly surface, and

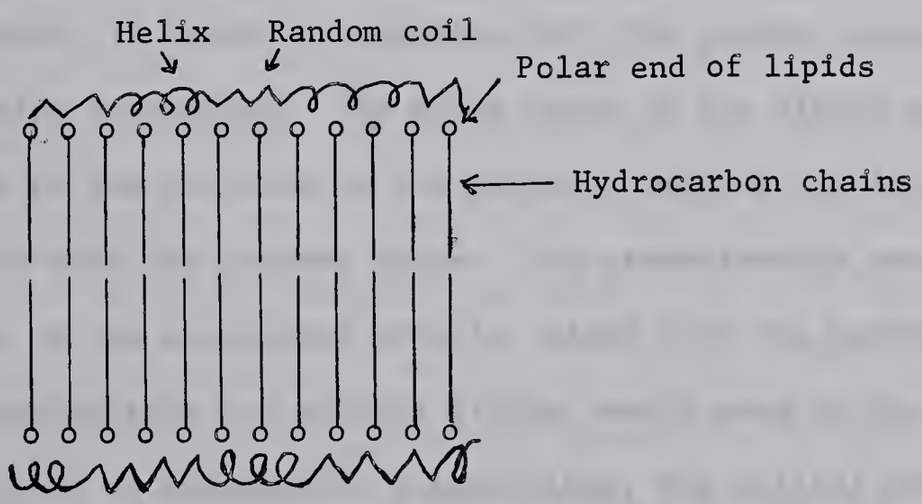
the diameters of the bumps are about 200 Å, which they interpreted as plaques lying on the red cell surface. Their work received some criticism, in that this pebbly surface may be an artifact; but later work by other investigators using the carbon replica technique demonstrated that the surfaces of osmium oxide-fixed, air-dried red blood cells and frozen-dried red blood cells have the same type of pebbly or granular surface texture. Glaeser and coworkers (32), using the carbon replica technique, found that when all but a few hundred Å of cellular material are digested away, the carbon merely acts as a rigid frame supporting the structure. One may still see some bumps, but most of the picture is composed of strand-like structures with bends or loops. Some of these strands seem to follow the surface contour, while others cross obliquely. These strands are about 200 Å in diameter. They suggest that, if the strands are underlying structural elements, the pebbly appearance of shadowed replicas may be due to a looped configuration of the filamentous structures.

In recent years a large body of information has been accumulated, which exposes the inadequacy of the Danielli-Davson model as a basis for the versatile biological activity of the membrane of the living cell. Under the Danielli-Davson model of ultrastructure, the lipid layer would determine not only the membrane's structure, but its biological function as well. Lipids alone would be responsible for carrying solutes into and out of the cell. Since many of the transport phenomena could not be explained by simple diffusion, it was required to postulate the presence of some special catalytically active sites, which were described as shuttles, pores, carriers, etc. Although Danielli also considered the

possible interaction of amino acid side-chains of proteins with the lipid in the membrane (30), this interaction was suggested to involve fully extended films of protein at the lipid surface, bound to the phospholipid layer essentially by ionic interaction with the hydrophilic groups of the phospholipids. It has been shown however by several authors (33-35) that the predominant binding mode is actually hydrophobic rather than electrostatic.

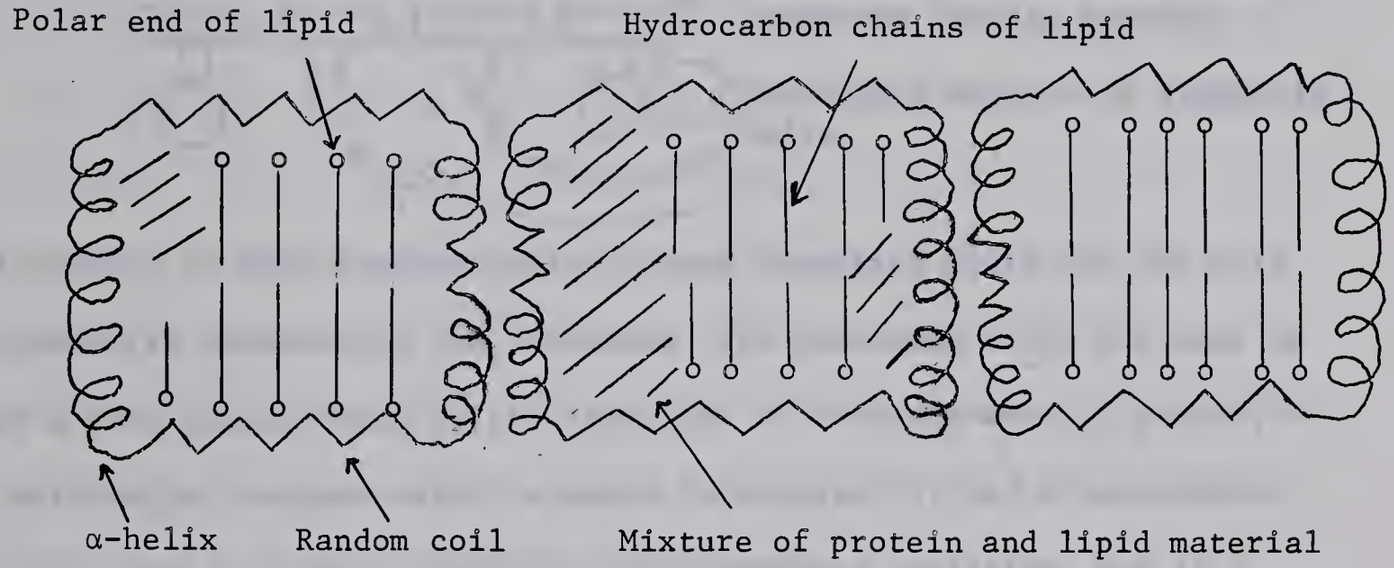
Lenard and Singer (36) established ORD and CD spectra of intact red cell ghosts, and they have calculated that one-quarter to one-third of the membrane protein is in the helical configuration, while the remainder is probably in the random coil form. They suggested that the spectra may be largely due to structural proteins, which would be expected to exhibit similar conformational characteristics and interactions in different membranes. These results are rather hard to reconcile with the Danielli-Davson model. By incorporating in this model the structural protein with its one-third α-helical content, a membrane shown in diagram 3 would be obtained.

Diagram 3.



Current concepts about proteins and other macromolecules cannot, however, be reconciled with this model. It is known that hydrophobic interactions play an important role in determining the conformation of globular proteins, and it has been suggested that the membrane structure in the native state is also determined by hydrophobic, rather than by electrostatic interactions. An alternative scheme as proposed by Lenard and by Korn (36,37) is indicated in diagram 4.

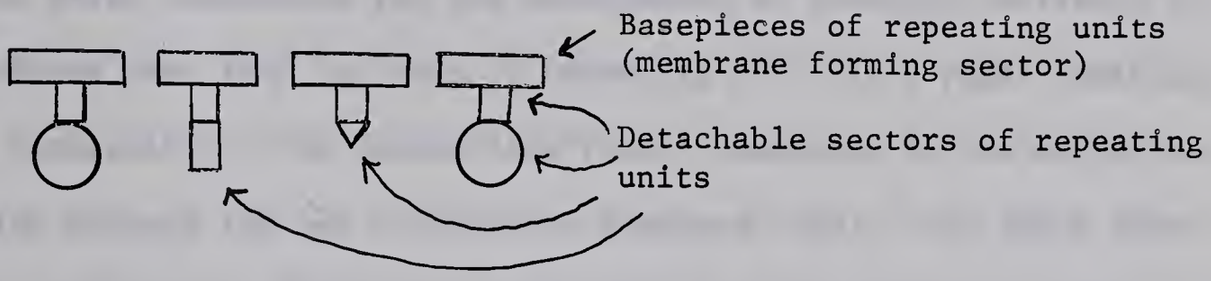
Diagram 4.



Although information at the present time is insufficient to validate the model, it is more consistent with the present knowledge of macromolecular structures. The polar heads of the lipids and the ionic side chains of the proteins on the membrane surface are in van der Walls contact with the aqueous phase. The predominantly non-polar side chains of the structural protein, along with the hydrocarbon portions of phospholipids and neutral lipids, would make up the interior. Stabilized by hydrophobic interactions, the helical portions of the proteins were located in the interior. This type of structure could

form an intact membrane, by aggregating in two dimensions. It would reconcile the present day concept, that complex macromolecular structures are build up of smaller repeating units. According to Green's concept (38,39), the membrane would be built up of nesting lipoprotein repeating units, the diagrammatic representation of which is shown in the illustration below.

Diagram 5.



According to this interpretation, these repeating units are the only structural elements in the membrane. The repeating units are made up of a base piece, which is the invariant or membrane-forming sector, (which might compare with the units in diagram 4), and a detachable sector, which is not essential to the membrane continium, but is a projection therefrom. Although the detachable sectors are an intrinsic part of the membrane, their presence is not essential to the maintenance of the membrane's structure. The repeating units of the membrane are complementary in form only, while in composition they may be different from each other, each chemically and enzymically unique.

The modern architectural anatomy of the membrane just presented would give the proteins a more active role in the many diversified tasks with which the membrane is concerned. In recent years evidence has accumulated indicating that the erythrocyte membrane contains a

number of enzyme systems that play a role in active transport, as well as others participating in metabolic control. Active transport (which is referred to by the term "cation pump") is defined as the uphill movement of an ion, that is, movement against an electrochemical potential gradient. Thus, the pump specifically involves the exchange of K outside for Na inside the cell. The movement of ions by the mechanism of active transport requires the expenditure of energy.

The first indication for the involvement of catalytic activity in the membrane came from the work of Davson in 1942, in a paper entitled: "Ionic permeability: an enzyme-like factor concerned in the migration of sodium through the cat erythrocyte membrane" (40). Ten years later Clarkson and Maizels found that erythrocyte ghosts contain enzymes capable of hydrolyzing ATP and other phosphate esters on the erythrocyte surface (41). The linking of these enzymes with the active movement of Na and K across the erythrocyte membrane led to the field of studies of active transport and ATP-ase.

One of these ATP-hydrolyzing enzymes has been investigated in great detail and found to be responsible for the transport of monovalent cations across the erythrocyte membrane. This enzyme (ATP Phospho-hydrolase EC 3.6.1.3) often known as (Na + K)-ATP-ase seems to be integral part of the membrane and to consist of two components (84,85). The first component requires the presence of Mg ions and it is not inhibited by cardiac glycosides; the second component requires the presence of both Na and K in addition to Mg ions, and it is completely inhibited by cardiac glycosides at concentrations identical to those

which inhibit active transport. The enzyme system is dependent upon the presence of Mg as had been found previously for active transport. The activation of the second component by K in the presence of Na corresponds to the activation of the pump outflux of Na with extracellular K (86). Conversely, the Na activation of the enzyme in the presence of K yields a similar correspondence. Using the reversal of hemolysis technique (86,87) it was further shown, that the rate of ATP hydrolysis within the cell is dependent upon the initial Na concentration but only if K is present in the external medium. Both the external Na concentration and the internal K concentration, have no effect upon the rate of ATP hydrolysis. Thus it appears that the membrane ATP-ase is controlled by the internal concentration of Na and external concentration of K, the location from which these ions are actively transported. The relationship between the two components of the ATP-ase is still not clear, in fact it has been suggested that they may be two distinct enzymes (75); however the close correlation between the movement of ions across the membrane and (Na + K) - activated ATP-ase clearly suggests its involvement in active transport. Although the molecular mechanism by which the hydrolysis of ATP results in the active transport of Na and K ions is still obscure, several hypothetical mechanism have been suggested (88,89). The initial step in the transport of ions might involve a Na-dependent phosphorylation of an acceptor molecule (90) possibly the enzyme itself, followed by a K-dependent dephosphorylation (91).

The work of Gourley on phosphate transfer through the erythrocyte membrane (42,43) also indicated the involvement of glycolytic enzymes. By studying ^{32}P distribution in the acid-soluble phosphate compounds, he showed that phosphate ions enter the cell by the formation of ATP at the cell surface. Bartlett (44), following similar lines of investigation, postulated that the three glycolytic enzymes - glyceraldehydephosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) EC 1.2.1.12); phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3); and lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.11.1.a), - involved in the transfer of phosphate via ATP, are localized in the erythrocyte membrane. Although Bartlett failed to show the presence of these enzymes in the erythrocyte ghosts, at a later date Ronquist et al. reported the formation of ATP when a ghost suspension was incubated in the presence of substrates and cofactors for TPDH and PGK (45,46). A more direct proof for the presence of glycolytic enzymes - which were usually regarded as soluble cytoplasmic enzymes - came from the investigation of Schrier (4,6). He set up his experiments to determine whether the erythrocyte membrane contains enzymes that could provide energy for active transport, mediate transport, or act as carriers. He found the presence of a number of glycolytic enzymes in the ghost suspension, but the activities of these enzymes were lower, and did not parallel, the enzyme levels in the respective hemolysate. The finding of Schrier, that TPDH and PGK are indeed present in the membrane of the red blood cell, gives weight to Bartlett's (44) thesis, whereby

TPDH is the mediator of phosphate transport, across the cell membrane. His observation further suggests that energy for the active transport of Na and K is supplied in the membrane, through substrate level phosphorylation mediated by TPDH which couples with PGK resulting in ATP formation.

There is some evidence presented by Parker and Hoffman (7) whereby membrane-bound phosphoglycerate kinase may play a key role in bridging active transport to energy production of the erythrocyte. According to their proposal, the ATP-ase of the membrane would produce a compartmentalised form of ADP, which would specifically react with phosphoglycerate kinase of the membrane. Another membrane-bound enzyme, triosephosphate dehydrogenase, would complete the cycle of an ATP-generating system for the cation pump. The rate of the two-enzyme sequence (triosephosphate dehydrogenase and phosphoglycerate kinase) was found to be a function of the ADP concentration. Since the initial addition of ATP made the "forward" reaction sensitive to the inhibitory action of ouabain, a control exerted by the pump over the glycolytic rate of the erythrocyte was postulated.

Green and associates (3) found the complete glycolytic sequence to be operative in their membrane preparation, obtained from beef erythrocytes. They found a wide divergence in the firmness of association of individual glycolytic enzymes, the pattern of which seemed to be affected by slight modifications in the preparatory method of the membrane fraction. It was found that the over-all glycolytic activity per mg of protein in the stroma was concentrated 24 times, with reference to the same activity in the whole hemolysate.

They were able to extract, and subsequently recombine TPDH with the membrane. They concluded that, in the intact cell, the complete glycolytic complex of enzymes is associated with the membrane, and is not free in solution.

The association of several other enzymes has also been reported recently in the literature (4,5,47-50); these reports are however often conflicting with regard to the presence of certain enzymes as well as the type of association involved between a particular enzyme and the erythrocyte ghosts. While Green and associates (3) reported the presence of the complete glycolytic sequence in their membrane preparations, Schrier (4) found no glucose utilization by the erythrocyte ghosts, which he mainly attributes to the absence of hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) and triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1). Although Schrier was able to measure pentose consumption and formation of ketopentoses when he used ribose 5-phosphate as a substrate, the use of nucleosides as substrates yielded negative results. The inability of his membrane preparation to utilize inosine was shown to be due to the absence of phosphoribomutase. Schrier's results are in conflict with reports by Lionetti et al. (5), who were able to show phosphate ester production from inosine. This conflict Schrier attributes to differences in the two membrane preparations.

Similar conflicting results are obtained by comparing reports of different investigators on the type of association of catalytic proteins with the erythrocyte membrane. While some authors report

firm attachment of triosephosphate dehydrogenase (3,4,46) and aldolase (3,4) to the membrane structure of the erythrocyte, others negate this observation (47).

It was therefore of interest to examine various factors which may determine the association of enzymic activities with the erythrocyte membrane; to design experiments which could contribute to the understanding of the type of interaction between enzymes and ghosts; to see possible changes that would occur in the morphology of the cell membrane under these different conditions; to find out the kinetic response of some enzymes under different conditions; and finally to attempt to relate and evaluate the observations made here with those reported in the literature, in the hope that it may lead a step forward to the elucidation of the molecular ultrastructure of the erythrocyte membrane.

II. MATERIALS AND METHODS

All chemicals used in the course of this investigation were of reagent-grade. Enzymes, coenzymes, and substrates employed in the different assay systems were obtained commertially from Boehringer Mannheim Corp. (N. Y.) and from Calbiochem. (Los Angeles). Water used for the preparation of solutions was distilled, and was put through a double-bed resin in a Crystalab Deeminizer. The pH of each solution was measured on a Radiometer pH meter, Model PHM 26, and adjusted within $\pm$ 0.02 unit of the desired value by titration with hydrochloric acid or sodium hydroxide.

Osmolarity measurements on the buffer solutions were made by an Advanced Instruments osmometer, Model 31 L. which has a three digit read-out system. After the buffer solutions were prepared at the desired pH values, their osmolarity was determined and adjusted to give a reading of 80 mOsm on the osmometer. They were further diluted with water to obtain buffers with lower osmolarity, as required in the experiments. Finally the pH was adjusted again to the proper value, by mixing of two buffers, having the same osmolarity but different pH. Buffers with low buffering capacity were kept in polyethylene bottles and were used within a day of preparation.

Preparation of ghosts

Venous blood samples drawn into EDTA (1 mg per ml) from patients in the University of Alberta Hospital, Edmonton were obtained from the hematology laboratory. They were pooled and centrifuged in a MSE clinical centrifuge at 1400 g for 7 minutes. Serum and buffy coat

were aspirated. The packed erythrocytes were washed by resuspension in 3 vol. of 1% NaCl solution and were centrifuged for 7 minutes at 1400 g at room temperature. The washing was repeated two more times. The packed cells were resuspended in 1% NaCl solution to yield a 25% hematocrit. Samples of these erythrocyte suspensions were taken for counting in a Coulter model B counter. Subsequently 4.0 ml aliquots were transferred into 50 ml polycarbonate centrifuge tubes, where they were hemolyzed by the addition of 35 ml hypotonic buffer (14-16). The concentration, pH, and composition of the buffers varied with each experiment. The hemolysates were then centrifuged in a Lourdes centrifuge for 20 minutes at 9000 x g at room temperature. The supernatant was aspirated and the ghosts were washed 3 times in the corresponding buffer, by resuspending the ghosts in a total volume of 40 ml. The washed stroma was made up to a known volume with the corresponding buffer. Samples were taken of each ghost preparation for counting in the Coulter counter. Counts were found to check within $\pm$ 5% of each other in the particular preparation as well as with the original erythrocyte suspension.

Assay of enzymes

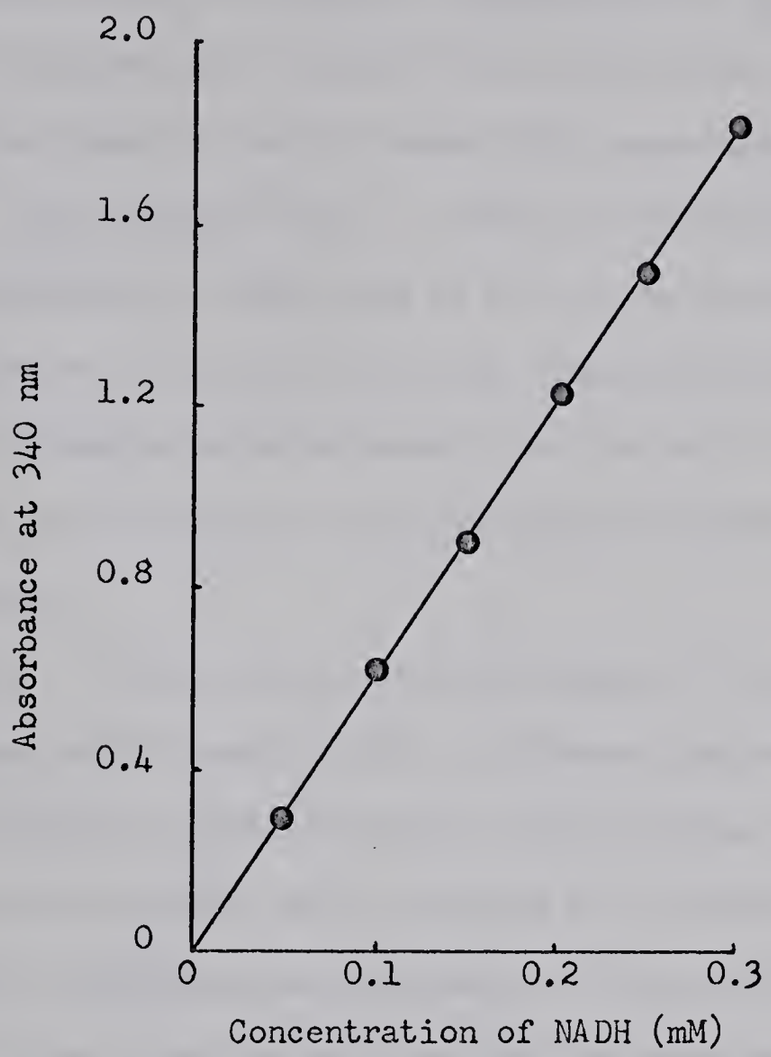
Unless otherwise indicated all the enzymes were assayed spectrophotometrically at 28°C in a 3 ml cuvette of a Beckman DU spectrophotometer, which was coupled to a Gilford automatic cuvette positioner, Model 200, optical density converter, Model 210, and absorbance indicator, Model 220 and a Sargent recorder, Model MR. The apparatus described gives a linear response up to 3.00 absorbance

units, and provides the possibility of setting any desired absorbance to give a 25 cm full scale deflection on the recorder. In the majority of the experiments, a value of 1.25 was used to give a full scale deflection on the recorder.

Unless otherwise specified, the total volume of the assay mixture was 3.0 ml, and the enzyme activities were defined as the number of micromoles of NADH or NADPH oxidized or NAD reduced per minute by 1.0 ml ghosts (IU per ml ghost). They were calculated on the basis of a molar absorbance of 6.22×10^3 for NADH and NADPH at 340 nm (51). The conversion of coenzyme during the enzymic reactions was followed by continuous recording of the change in absorbance of the system at 340 nm after initiation of the reaction, employing a cuvette of 1.0 cm light-path.

In order to establish how much change in absorbance may take place due to autoxidation of NADH, an assay mixture was put up for each enzyme of interest. One of the ingredients required for the reaction to proceed, however, was left out of the cuvette, for each and every enzyme in question. Recording for 20 mins. - which was longer than the time used for any of the enzymic reaction - revealed no significant change in the absorbance of the systems, apart from the case of one enzyme, which will be dealt with in more detail later.

Fig. 1. Standard curve of NADH concentration in the presence of ghosts



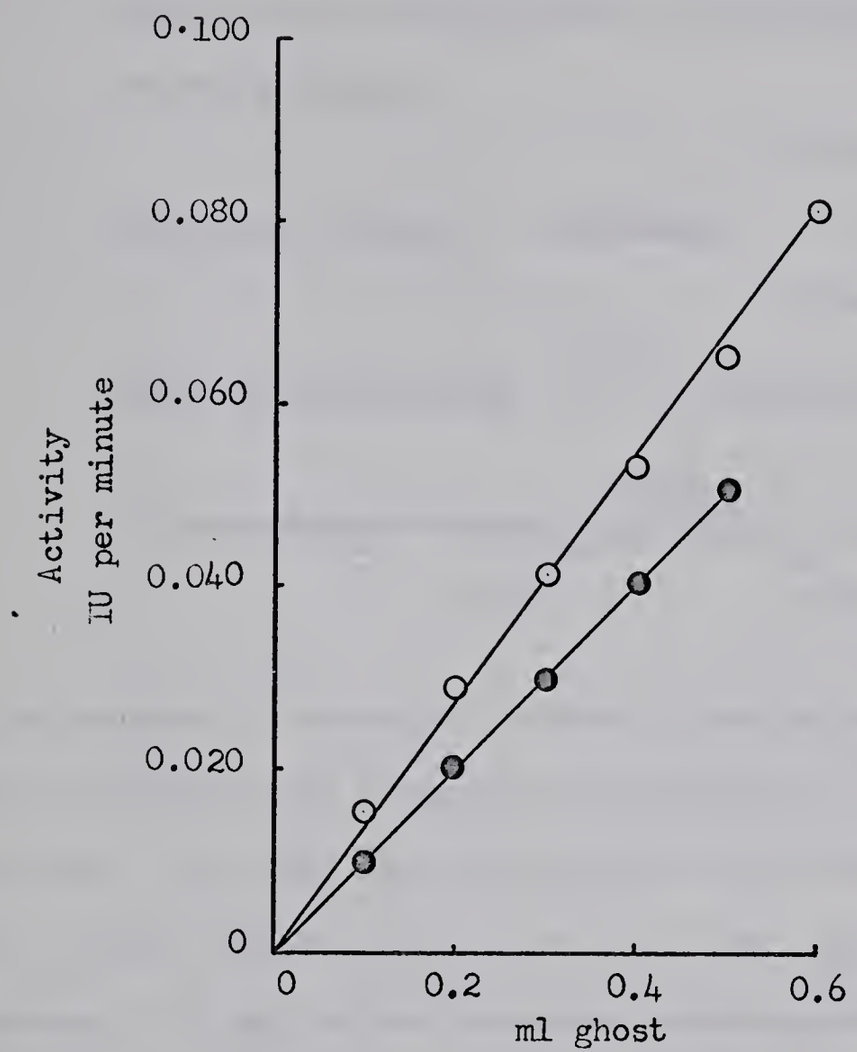
To aliquots of ghost suspension an increasing concentration of NADH solution was added, and the samples were brought to a final volume of 3.0 ml with H_2O.

To assure that changes in absorbance during the enzymic reactions truly reflect the changes in concentration of oxidized and reduced pyridine nucleotides, increasing concentrations of the reduced coenzyme in the presence of ghosts were read in the spectrophotometer. A plot of absorbance at 340 nm versus NADH concentration resulted in a straight line relation (Fig. 1), when the concentration was varied up to the concentration of NADH used in any of the assay systems. A slight deviation of the curve from the theoretical slope may be attributed to some adsorbed moisture from the air; to some degree of autoxidation and to the fact that NADH is not available commercially in 100% purity.

In order to balance the spectrophotometer, it was necessary to make up blanks which absorb light to different degrees at 340 nm. Cyanmethemoglobin in three different concentrations was sealed into glass stoppered cuvettes, which, because of its stable color, served as attenuator throughout the experiments. It was only required to take one of these cuvettes which matched for absorbance closest to the samples, and use it as an attenuator. This arrangement served very satisfactorily as an attenuator system, and was justified, since enzymic activities were expressed on the basis of changes in absorbance per unit time.

Conditions were arranged whereby the enzyme to be measured was rate-limiting. The number of ghosts per assay mixture was kept within limits, where the velocity was proportional to the enzyme concentration. A representative graph pertaining to pyruvate kinase and aldolase is shown in Fig. 2.

Fig. 2. The effect of the volume of ghost suspension upon the pyruvate kinase and aldolase activity of erythrocyte ghosts



Ghosts were prepared in 30 mOsm Tris buffer, pH 7.4, and were suspended in water prior to the addition of assay mixture. (o) pyruvate kinase; (●) aldolase.

1. Aldolase was assayed either a) by the method of Schrier (4) in which the reaction is coupled with NAD-dependent triose phosphate dehydrogenase; 2 equivalents of NADH are produced for each fructose-1,6-diphosphate reacting, in the following manner:

fructose-1,6-diphos. $\xleftarrow{\text{aldolase}}$ glyceraldehyde-3-phos. + dihydroxyacetone-phos.

dihydroxyacetone-phos. $\xleftarrow{\text{TPI}}$ glyceraldehyde-3-phos.

2 glyceraldehyde-3-phos. $\xrightarrow{\text{TPDH}}$ 2 1,3-diphosphoglycerate

2 NAD → 2 NADH

In the presence of arsenate 1-arseno-3-phosphoglycerate is formed, which is unstable and spontaneously hydrolyses to 3-phosphoglyceric acid (53). The assay mixture contained Tris buffer, pH 7.4, 50 mM; NAD, 1.66 mM; sodium arsenate, pH 7.4, 17 mM; triose phosphate isomerase, 0.01 mg; triose phosphate dehydrogenase, 0.1 mg; and the reaction was initiated by the addition of fructose-1,6-diphosphate, 5 mM.

b) By the method of Wu and Racker (52), whereby the reaction is coupled with triose phosphate isomerase and NADH dependent α-glycerophosphate dehydrogenase. In the reaction two equivalents of NADH are oxidized for each fructose-1,6-diphosphate reacting, according to the following scheme:

fructose-1,6-diphos. $\xleftarrow{\text{aldolase}}$ dihydroxyacetone-phosphate + glyceraldehyde-3-phosphate

glyceraldehyde-3-phos. $\xleftrightarrow{\text{TPI}}$ dihydroxyacetone-phosphate

2 dihydroxyacetone-phos. $\xrightarrow{\alpha\text{-GPDH}}$ 2 glycerophosphate

2 NADH — 2 NAD

The assay mixture contained Tris buffer, pH 7.4, 50 mM; NADH, 0.33 mM; triosephosphate isomerase, 0.01 mg; α-glycerophosphate dehydrogenase, 0.01 mg; and fructose-1,6-diphosphate, 2 mM.

2. Triose phosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) was assayed in the reaction, glyceraldehyde-3-phosphate ⟶ dihydroxyacetone phosphate, by coupling with NADH-dependent α-glycerophosphate dehydrogenase (54) in a system containing Tris buffer, pH 7.4, 50 mM; NADH, 0.33 mM; α-glycerophosphate dehydrogenase, 0.01 mg; and the reaction was initiated by the addition of DL-glyceraldehyde-3-phosphate, 4 mM.

Since only the insoluble barium salt of glyceraldehyde-3-phosphate is available commercially, it had to be converted into soluble sodium salt, prior to use. The method (55) adopted is as follows: In a pyrex test tube BIO-RAD analytical grade cation exchange hydrogen-form sulphonate resin (stock No. AG 50 w-X8) was suspended in deionized water, to which DL-glyceraldehyde-3-phosphate diethylacetal barium salt was added. The tube was placed in boiling water bath for three minutes and was shaken intermittently. It was then cooled by transferring the tube to an ice bath, followed by centrifugation and careful removal of the supernatant. The resin was washed in small

aliquots of water several times for extraction of the free DL-glyceraldehyde-3-phosphoric acid into the supernatant fluids, which were combined and made up to volume to obtain the desired concentration. The pH of the solution was around 2. Prior to use, aliquots of the solution was neutralized by addition of sodium bicarbonate, while the remaining portion was kept frozen in 1 ml. aliquots.

3. Triose phosphate dehydrogenase was assayed by combining the methods of Racker (52) and Schrier (4). The assay mixture of the reaction glyceraldehyde-3-phosphate ⟶ 3-phospho-glyceric acid, contained Tris buffer, pH 7.4, 50 mM; sodium arsenate, pH 7.4, 5 mM; NAD, 1.3 mM; nicotinamide, 30 mM; aldolase, 0.1 mg per cuvette, and fructose-1,6-diphosphate, 5 mM. Since the rate of reaction falls sharply after 1-2 min, the initial rates were determined from the recording between 15 and 60 secs. after initiation of the reaction.
4. Phosphoglycerate kinase was assayed in the "backward" reaction as described by Schrier (4), through the conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate by coupling with NADH-dependent triosephosphate dehydrogenase. The reaction mixture contained: Tris buffer, pH 7.4, 50 mM; $MgCl_2$, 10 mM; GSH, 5 mM; ATP, 2 mM; NADH, 0.33 mM; triose-phosphate dehydrogenase, 0.1 mg per cuvette, and the reaction was initiated by adding 3-phosphoglycerate, 3.3 mM. Presence of 2,3-diphosphoglycerate, 5 mM, did not affect the measured activity.

5. Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) was assayed in a system (56) where the conversion of phosphoenol pyruvate ⟶ pyruvate is coupled with NADH-dependent lactate dehydrogenase. The reaction mixture contained: Imidazole-Histidine buffer, pH 7.4, 25 mM; KCl, 75 mM; $MgCl_2$, 8.3 mM NADH, 0.33 mM; phosphoenol pyruvate, 3 mM; ADP, 2 mM; and lactate dehydrogenase, 0.05 mg per cuvette.

6. Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) was determined by measuring the rate of oxidation of NADH (57). The assay mixture contained: Tris buffer, pH 7.4, 60 mM; NADH, 0.33 mM, sodium pyruvate, 0.6 mM.

7. Glutathione peroxidase (glutathione: H_2O_2 oxidoreductase, EC 1.11.1.a) was assayed as described by Paglia et al. (58). This method measures the rate of GSH oxidation by H_2O_2 as catalyzed by the GSH-peroxidase present in the red blood cell membrane. Rather than measuring progressive loss of GSH however, this substrate is maintained at constant concentration by the addition of GSSG-reductase (NADPH: glutathione oxidoreductase, EC 1.6 U 2), and NADPH, which converts any GSSG produced to the reduced form:

$$2\ GSH + H_2O_2 \xrightleftharpoons{GSH\text{-}P} H_2O + GSSG \xrightleftharpoons{GSSG\text{-}R} 2\cdot GSH$$

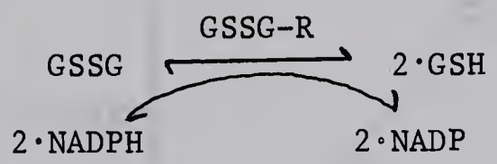
$$2\cdot NADPH \qquad\qquad 2\cdot NADP$$

The rate of GSSG formation is then measured by following the oxidation of NADPH spectrophotometrically. The reaction mixture

contained: Phosphate buffer, pH 7.4, 80 mM; NADPH, 0.33 mM; GSSG-reductase, 0.05 mg per cuvette; sodium azide, 20 mM; GSH, 5 mM; and the reaction was initiated by addition of H_2O_2, 0.07 mM.

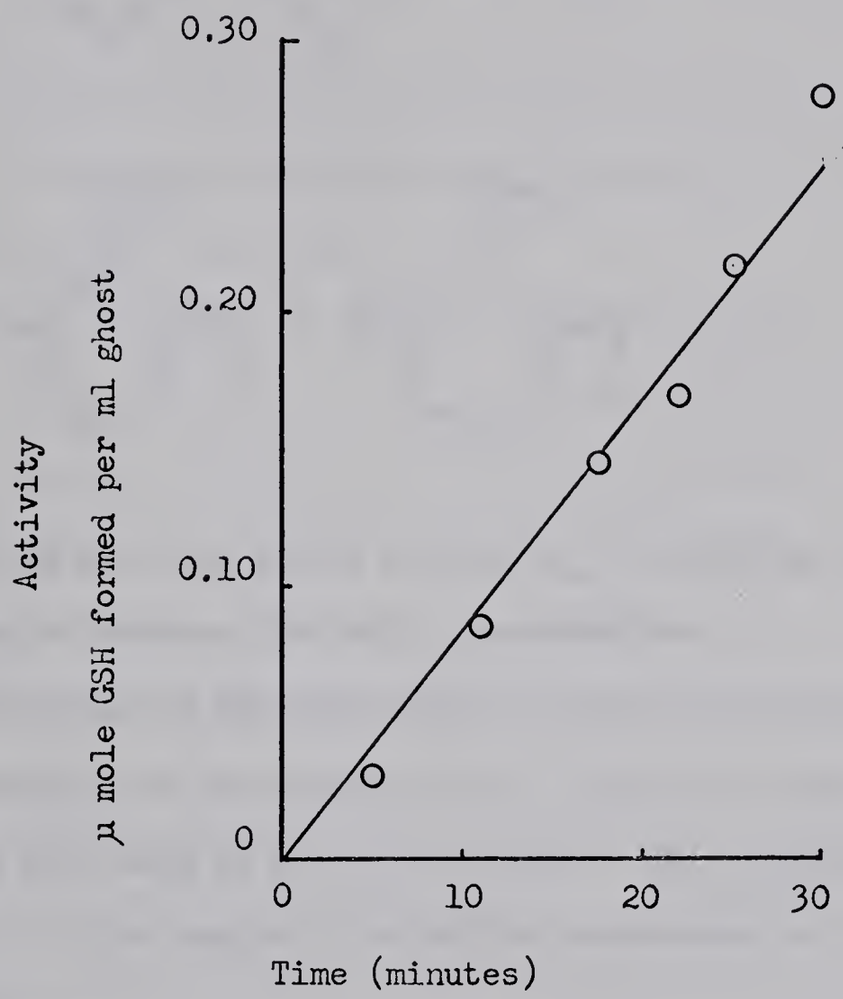
The nonenzymic oxidation of GSH was determined by simultaneous assay of the system identical to the enzymic oxidation, except for replacement of the ghosts by an equal volume of water. The reaction rate of the non-enzymic oxidation system was substracted from that of the total oxidation to determine the true enzymic activity.

8. Glutathione reductase activity was determined in the reaction,



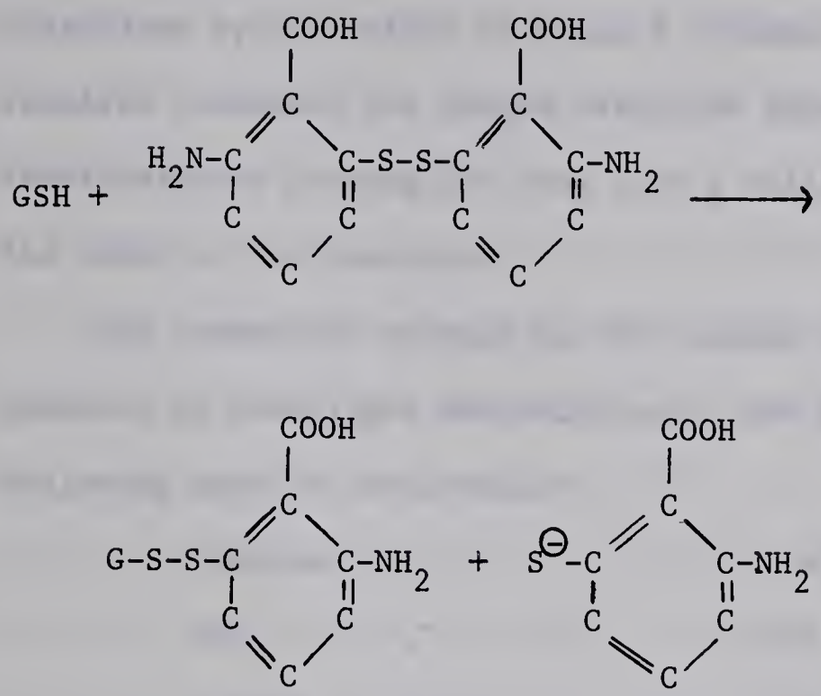
by measuring the concentration of GSH formed during 20 mins. incubation at 37° (59). The assay mixture contained: Tris buffer, pH 7.4, 50 mM; NADPH, 0.5 mM; serum albumin, 0.03% w/v; and neutralized GSSG, 0.5 mM. Appropriate blanks were set up by the use of inactivated enzyme. The reaction was stopped by NaCl-saturated 2% metaphosphoric acid solution, which was added in the ratio of 2:1. The protein precipitate was filtered off, using Munktell's No. OK filter paper, and the filtrate was used for determination of GSH concentration. The method of assaying GSH was the modification of Beutler (60), whereby GSH is reacted with excess of DTNB (5,5'-Dithiobis-(2-nitrobenzoic acid)) in TEA (2,2',2-Nitrilotriethanol)

Fig. 3. Progress curve of glutathione reductase of erythrocyte ghosts.



Ghosts were prepared in Tris buffer, pH 7.4, and suspended in water prior to the addition of the assay mixture.

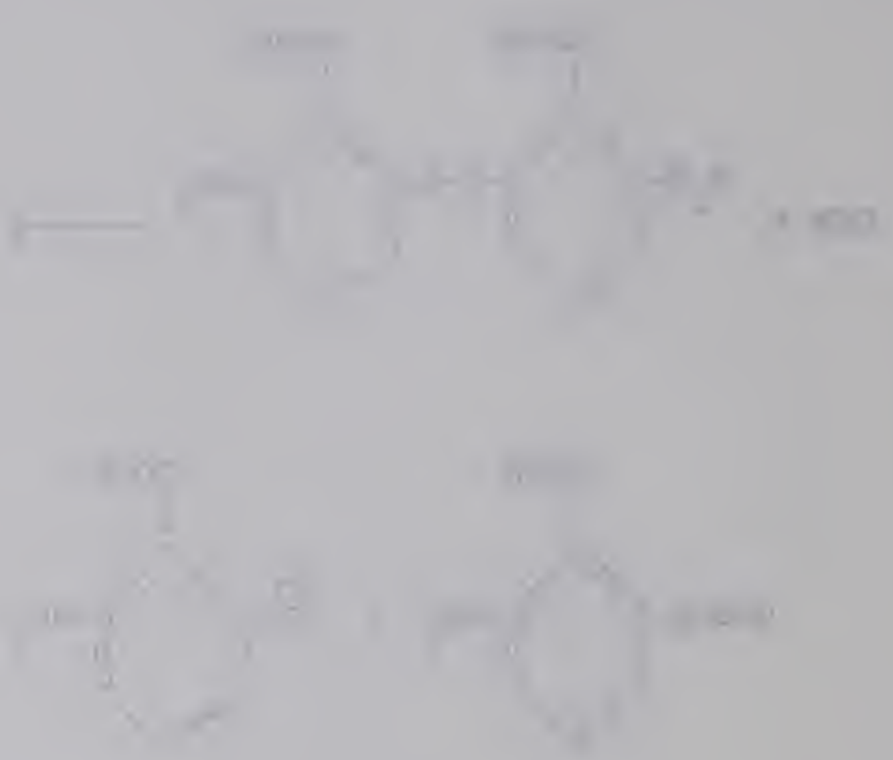
buffer, pH 7.5, 0.66 mM, in the following fashion:



The resulting anion is highly colored (ε_m = 13,000 at 412 nm) and it can be used to measure the thiol concentration.

The velocity of GSSG-reductase was found to be linear with time up to 30 minutes as indicated in Fig. 3, which is longer than the incubation time used in the enzyme assays. The linearity of reaction velocity with time assured that initial velocities of the enzyme were determined.

9. ATP-ase activity was assayed in the reaction ATP $\longrightarrow$ ADP + P_i, by determination of inorganic phosphate produced in the enzymic reaction. The assay mixture for ATP-ase (61) contained: Tris buffer, pH 7.4, 30 mM; NaCl, 100 mM; KCl, 16 mM; MgCl, 3.0 mM; Ouabain where necessary, 0.1 mM; and ATP, pH 7.4, 2 mM. The assay was carried out at 37° for 30 minutes and was stopped by addition of ice-cold TCA in a



final concentration of 5% w/v. The protein precipitate was centrifuged off and the inorganic phosphate in the supernatant was determined by the method of Fiske & SubbaRow (62). The blanks required contained the system described above, but the enzyme was inactivated by placing the tube into a boiling water bath prior to the start of the reaction.

The commercial enzymes in the coupled reactions were the products of Boehringer Mannheim Corp., New York, N. Y. and had the following specific activities:

Aldolase	9 units/mg.
TPI	2400 units/mg.
α-GPDH	36 units/mg.
TPDH	36 units/mg.
LDH	360 units/mg.
GSSG-R	70 units/mg.

Determination of the hemoglobin content of ghosts was carried out by the method of Kampen et al. (63). The method is dependent on the fact, that all common hemoglobin derivatives can readily be converted into methemoglobincyanide. This stable colored compound has a $\varepsilon^{540}_{meq} = 11.0$, using the equivalent weight of 16,114 for hemoglobin, calculated from the known molecular structure. A sample of ghost suspension was solubilized with sodium lauryl sulfate (1% w/v), and was added to Kampen's solution which contained the following: $K_3Fe(CN)_6$, 200 mg; KCN, 50 mg; KH_2PO_4, 140 mg; Sterox SE, 0.5 ml and water to 1.00 l.

Protein was measured by the biuret method (64,65). This analysis is based on the formation of a purple-blue color, when proteins are reacted with alkaline copper sulfate solution. The color was read spectrophotometrically at 555 nm against Lab-Trol, a commercial protein standard.

Cholesterol was extracted at room temperature into a 3:1 mixture of alcohol and ether, as described by Varley (66). After centrifugation however the precipitate was washed two-more times in the same mixture. The cholesterol-containing supernatants were pooled and evaporated to dryness in a rotary evaporator. The cholesterol was taken up in chloroform and determined either A) by the Liebermann - Burchard reaction, whereby cholesterol is reacting with acetic anhydride and sulfuric acid reagent, to form a cherry-red colored solution, which was read spectrophotometrically against a cholesterol standard at 680 nm. B) Cholesterol was determined by gas-chromatography through the courtesy of Mr. James Lo, who was working out a method for cholesterol.

Measurements of micro-hematocrit volumes of erythrocyte ghost were obtained as described by Hawk et al. (67). After the ghost suspensions were drawn up into blood collecting capillary tubes, one end of the tubes were sealed by flame. Subsequently they were centrifuged in an IEC clinical centrifuge at 5100 g for 30 minutes, using the 927 Hemato-Kit centrifuge head. The hematocrit was expressed as mm packed ghost per mm suspension.

Morphological studies of erythrocyte ghosts were carried out by a Zeiss phase contrast microscope at 250-fold magnification. The photomicrographs were recorded on Kodak Panatomic X 35 mm roll film.

Subsequent magnifications were obtained through an Opematus dark-room enlarger, and recorded on Kodabromide F-4 enlarging paper.

III. RESULTS AND DISCUSSION

Osmotic studies

The recognition of cytoplasmic contaminants in the erythrocyte ghost preparation is the most difficult problem in the isolation of pure membrane fractions. The intricacies can be best seen by the dilemma presented by hemoglobin. Whether hemoglobin is a true membrane component or a cytoplasmic contaminant is debatable at the present time. While in ghosts prepared at pH 6.0, hemoglobin may constitute up to 50% of the dry weight, it is completely removed by hemolysis at a higher pH (14,16). However, the removal of hemoglobin from the erythrocyte ghost by the use of higher pH (7.8 - 8.5), is accompanied by the removal of non-hemoglobin constituents from the membrane fraction (14,68). Although these ghosts appear to be unfragmented in the light microscope (14), Anderson and Turner (68) claim that the use of higher pH leads to the partial disintegration of the membrane. In addition to the pH effect, the ionic strength of the hemolyzing solution was also reported to have a profound role, in determining the final hemoglobin content of the ghost composition. Dodge *et al.* (14) found that the removal of hemoglobin was maximal at buffer concentrations between 10 and 20 ideal milliosmolar, while an increasing amount was retained below and above these buffer concentrations. The removal of hemoglobin was found once again to be accompanied by the removal of other non-hemoglobin proteins from the erythrocyte ghosts. Mitchell *et al.* (47) attempted to identify the nature of these non-hemoglobin proteins, which were subject to removal from the erythrocyte

ghosts due to changes in pH, or osmotic strenght of the hemolyzing buffer used in their preparation. While they were able to show that part of these non-hemoglobin proteins were enzymic in nature, some of their results are hard to explain, while others are contrary to reports by other investigators (3,4,6,7).

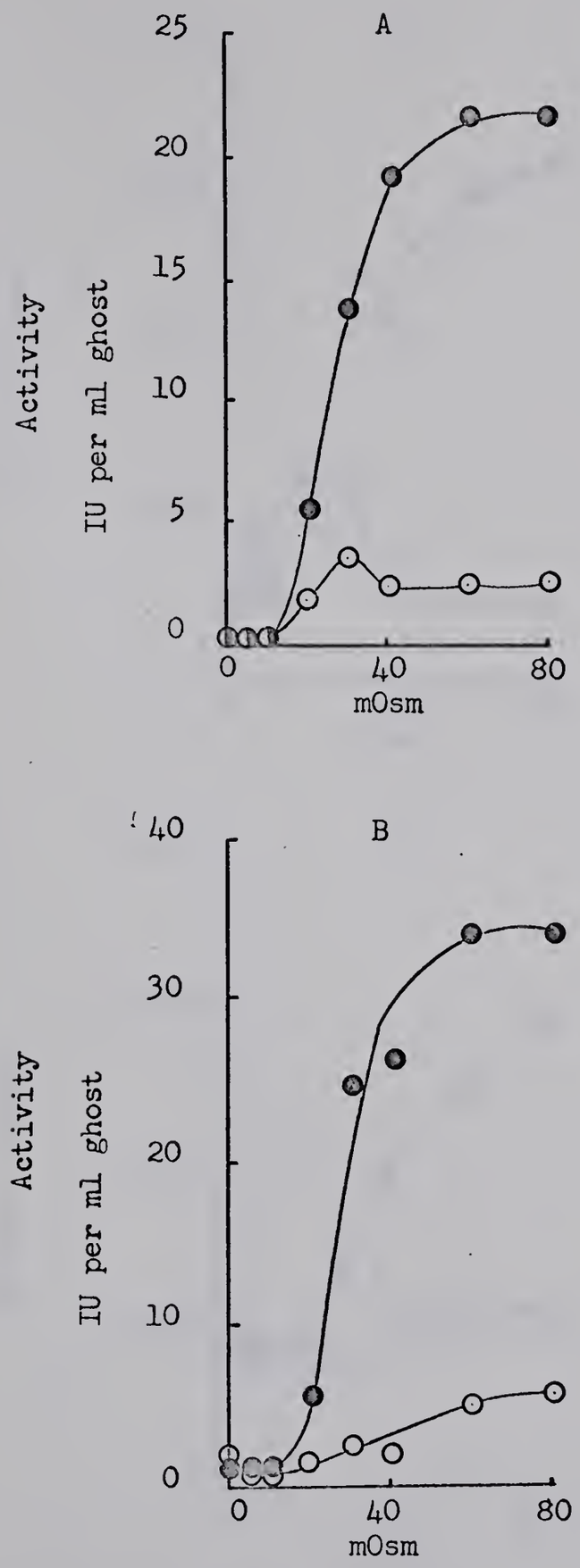
It seemed reasonable to reexamine the effects of osmotic strength and pH of the hemolyzing buffer on the final composition of the erythrocyte ghosts, in order to gain knowledge about the type of interactions between the erythrocyte membrane and some of the membrane-bound enzymes.

The effect of the osmotic strength of the hemolyzing buffer on the retention of enzymes by the erythrocyte ghosts was studied at constant pH. Aliquots of a washed erythrocyte suspension were hemolyzed and subsequently washed - as described under Methods - in Tris buffers, pH 7.4, which were hypotonic and had osmotic strenghts of 80, 60, 40, 30, 20, 10, 5, and 0 mOsm. The final volume of each ghost preparation was made up to the initial volume of the erythrocyte suspension, using the appropriate buffers, so as to have the same number of ghosts per unit volume in each preparation. The ghost suspensions were then tested for enzyme activities under two sets of conditions. In the first set of experiments, aliquots were diluted six-fold in the corresponding buffers prior to addition of the reaction mixtures, while in the other set of experiments water was used as a diluent. Representative data obtained from these experiments are presented in Figs. 4 - 7. These results demonstrate that the osmolarity of the washing fluid markedly influences the enzymic activity of the ghost preparation.

Perhaps the most obvious observation one can make on examination of these figures is the large difference in enzymic activities obtained under the two different conditions. Each and every enzyme tested showed a large increase in activity, when water was used as a diluent, over the activity obtained in the appropriate buffer, that is, as the osmolarity of the washing fluid was increased. The enzymic activity obtained when the erythrocyte ghosts are suspended in the corresponding buffer will hereafter be referred to as the "basic activity"; while the term "cryptic activity" will be used to signify the enhanced activity obtained when water is used as the suspending medium.

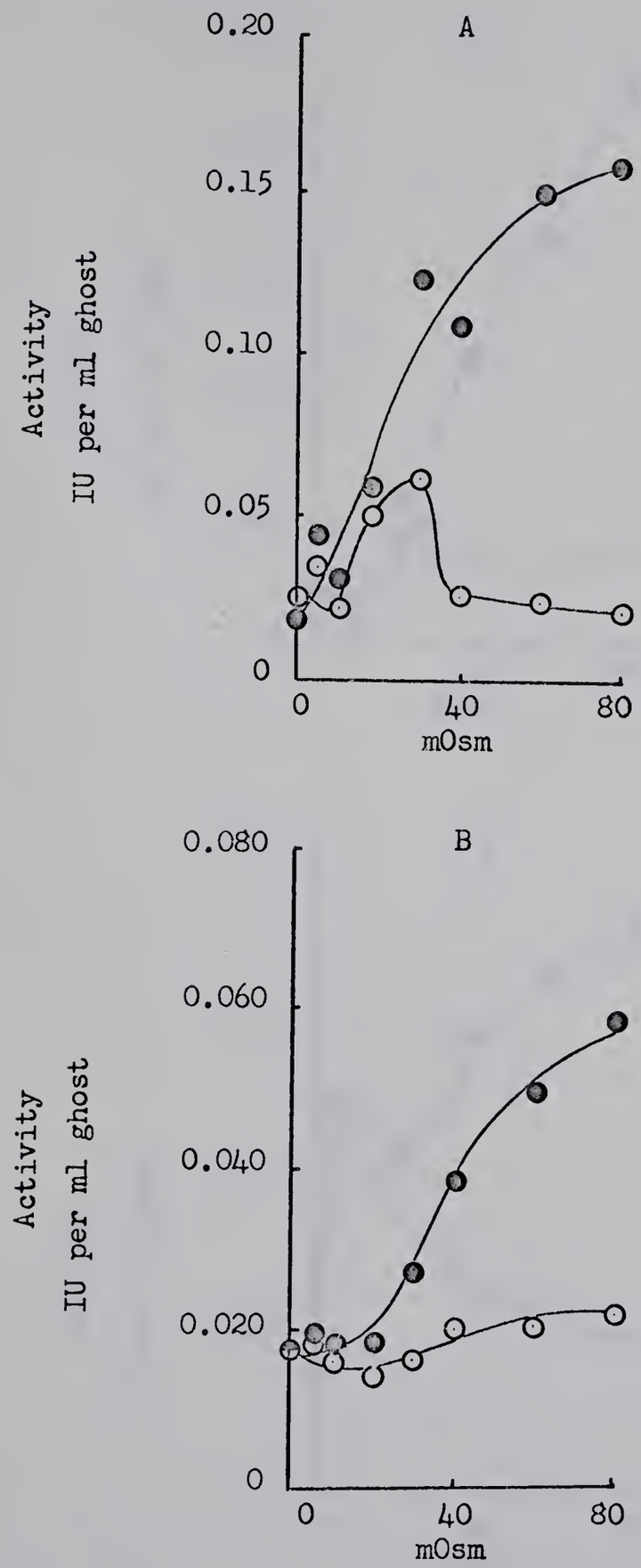
On the basis of the activity profiles - obtained by varying the osmolarity of the washing fluid - it is possible to classify these enzymes into three groups. Of the enzymes tested, group No. I includes LDH, TPI, PK, GSH-P, and GSSG-R. This group is characterized by the fact that the enzymic activities are minimal when buffers of low concentrations are used in the ghost preparation, and the retention of enzymes is maximal - especially the cryptic activities - when higher buffer concentrations are employed in the preparation of the ghosts. The opposite is true of group No. II which includes aldolase and TPDH. The activities of the enzymes are maximal at the lowest buffer concentration, while increasing the concentration of the washing fluid results in decreased enzyme activities. This is most apparent from inspecting the basic activities. PGK can not be included in either group described above, but rather it is a mixture, having characteristics of both categories. Its basic activity profile closely

Fig. 4. Dependence of enzyme retention by erythrocyte ghosts on the osmolarity of the washing fluid.



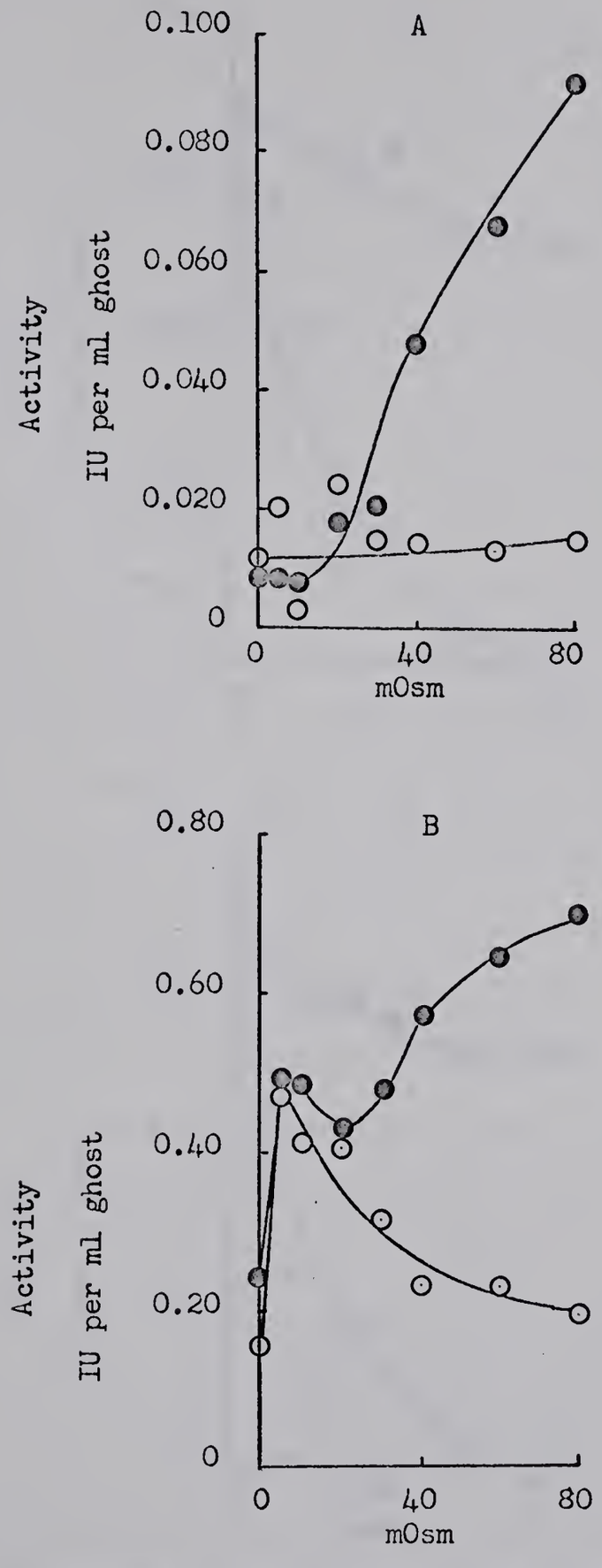
Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. The ghosts were diluted six fold prior to the addition of the assay mixture (o) in the corresponding buffer; (•) in deionised water. (A) lactate dehydrogenase; (B) triose phosphate isomerase.

Fig. 5. Dependence of enzyme retention by erythrocyte ghosts on the osmolarity of the washing fluid.



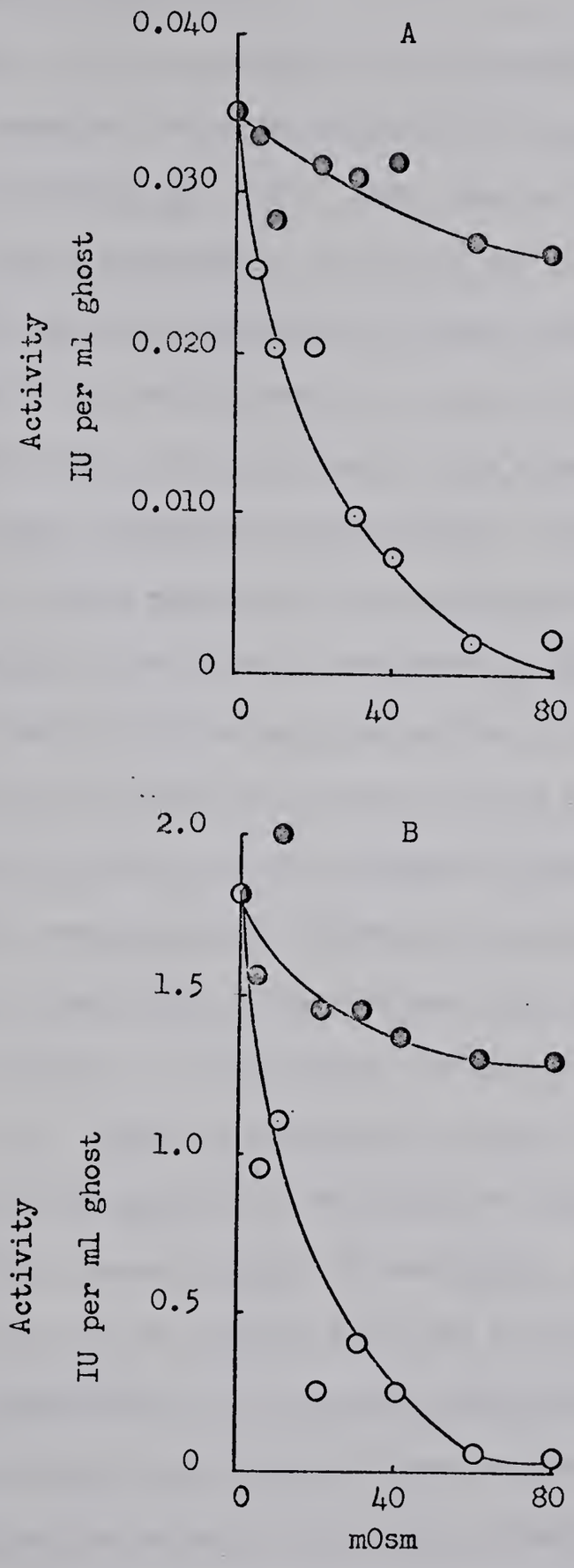
Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. The ghosts were diluted six fold prior to the addition of the assay mixture (o) in the corresponding buffer; (•) in deionised water. (A) pyruvate kinase; (B) glutathione peroxidase.

Fig. 6. Dependence of enzyme retention by erythrocyte ghosts on the osmolarity of the washing fluid.



Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. The ghosts were diluted six fold prior to the addition of the assay mixture (o) in the corresponding buffer; (•) in deionised water. (A) glutathione reductase; (B) phosphoglycerate kinase.

Fig. 7. Dependence of enzyme retention by erythrocyte ghosts on the osmolarity of the washing fluid.



Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. The ghosts were diluted six fold prior to the addition of the assay mixture (o) in the corresponding buffer; (o) in deionised water. (A) aldolase; (B) triose phosphate dehydrogenase.

resembles that of group No. II; while its cryptic activity profile is similar to that of group No. I.

The cryptic activity profile of the enzymes listed in group No. I remarkably resembles the curve obtained for hemoglobin under similar conditions by Dodge et al. (14). They observed by phase optics that, although the ghost preparation is carried out under conditions where 100% hemolysis was to be expected, the ghost population at the higher osmotic strength was heterogeneous in respect to its hemoglobin content. They found that while some of the ghosts were pale in appearance, others contained an appreciable concentration of hemoglobin. This finding of Dodge suggested that morphological studies may lead to the understanding of the results presented in Figs. 4 - 7.

In the present study using phase optics it was possible to view the ghosts under identical conditions to those employed in the activity profile studies for the different enzymes. This investigation revealed marked morphological differences in ghosts, which were affected by the osmolarity of the washing fluid as well as the medium used for the dilution. At the higher concentrations (30 - 80 mOsm) of the washing fluid, when the dilutions were made in the corresponding buffers, the ghosts appeared to be intact as shown in plate I-A. They contained varying concentrations of hemoglobin, as reported by Dodge. As the osmolarity of the washing fluid was decreased, however, an increasing fragmentation of the ghosts was apparent until at 10 mOsm or lower concentrations intact ghosts became scarce. Alghouth a more detailed study on the effect of low ionic strength on the membrane integrity will follow in a later section, it is appropriately mentioned

here, that the fragmentation of the erythrocyte ghosts at a lower ionic strength of washing fluid was confirmed through a determination of particle-size distribution, using the Couleter counter.

When the different ghost preparations were diluted in water, fragmentation of the ghosts was dominant throughout the whole range of buffer concentrations used the experiments. (Plate III-A & B). The observation that the ghosts break up when the osmolarity of washing fluid is below 30 mOsm complements the report by Teorell (9), who found that erythrocyte ghosts lose their permeability properties, when the hemolysate proportion reaches 1:10. An understanding of the basic and cryptic activity profile curves for the enzymes of group No. I thus emerged. At low ionic strength of the wash medium, as the red cell membranes are breaking up, the enzymes are liberated into the hemolysate, and are lost in the supernatant during the subsequent washings. Since intact ghosts regain their semipermeability, the escape of the enzymes into the hemolysate, and thus their loss into the supernatant during the washing procedures, is in effect reduced.

In the case of the basic activity profile, the increase in enzyme activity is moderate, (in some cases: LDH and PK even decline), as the osmolarity of the washing fluid increases beyond 30 mOsm. This is probably due to the compartmentation of the enzyme in question from the coupling enzymes used in the assay, by the semipermeable membrane of the intact erythrocyte ghost. Thus the movement of substrates between the enzyme molecules is hindered by the membrane, which may have to be crossed twice in order to detect the particular enzyme activity.

The cryptic activity profiles then can be explained as breaking the permeability barrier, by decreasing the ionic strength of the suspending medium prior to the enzyme assay. As a result the activity of the enzymes is greatly enhanced, as 30 mOsm or higher concentrations of the washing fluid are reached.

It was of interest to determine whether the cryptic activities are still retained by or liberated from the fragmented erythrocyte membranes. A stock ghost suspension was therefore prepared in 80 mOsm Tris buffer, pH 7.4, of which aliquots were removed and treated as follows. I. the aliquot served as a control without further treatment. II. the aliquot was diluted ten-fold in the same buffer that was used in the preparation of the stock ghost suspension. III. the aliquot was diluted ten-fold in distilled water. Tests No. II & III were centrifuged at 10,000 g for 30 minutes. The supernatants were aspirated and used in the subsequented enzyme assay with no further treatment. The recovered ghosts along with the control ghosts were tested for enzyme activities under two sets of conditions. In experiment A, a six-fold dilution of the ghosts was made in 80 mOsm Tris buffer prior to the enzyme assay, while in experiment B, water was used as a diluent. The results obtained are presented in tables I to III.

It can be seen from table II that the total enzyme activities obtained under the different conditions are in close agreement with each other. Table I reveals that the control ghost suspension as well as the ghosts washed in buffer exhibit both the basic and cryptic enzymic activities, while ghosts washed in water lack these differences.

Ghosts washed in buffer liberated varying amounts of enzyme activities to the supernatant; however, the majority of their activities were retained by the membrane fraction. Washing the erythrocyte ghosts with water, however, yielded no such uniform result. On the basis of the distribution of enzymic activity between the supernatant and the membrane fraction (Table III), it is possible to classify the enzymes into two groups. Those enzymes that have liberated most of their activities into the supernatant fraction form group A. This group includes GSSG-R, GSH-P, TPI, PK, and LDH, which enzymes will be referred to as "loosely-bound enzymes". Group B is characterized by the retention of a high proportion of the enzymic activity by the membrane fraction. This group, whose enzymes will be called "firmly-bound", is represented by TPDH and aldolase. PGK was an intermediate between the two groups, since it has liberated half of its activity into the supernatant, while the other half was retained by the membrane fraction.

Referring back to the grouping of the enzymes according to their activity profile, it is evident that the enzymes coincide member for member with the present grouping. This would indicate an interrelation between the nature of the binding of an enzyme to the erythrocyte ghost and its activity profile. This interrelation subsequently would be useful in explaining the type of activity profile obtained by the firmly bound enzymes of the erythrocyte ghost.

When the preparation of the erythrocyte ghosts is carried out in a low mOsm buffer, the membrane - as described before - is broken up. Consequently a certain portion of the interior structure is brought in

Table I. Distribution of enzyme activities of the erythrocyte ghosts between membrane and supernatant fractions, as affected by the osmolarity of the wash medium.

Sample		Enzyme activity IU per ml fraction							
		GSSG-R	GSH-P	TPI	PK PK	LDH	PGK	TPDH	Aldolase
Control ghost suspension	In buffer	0.080	0.009	3.63	0.004	0.31	0.10	0.14	0.035
	In water	1.15	0.059	11.6	0.027	1.94	0.54	0.40	0.075
Ghosts washed in 80 mOsm Tris buffer, pH 7.4	In buffer	0.050	0.003	0.92	0.002	0.16	0.09	0.12	0.030
	In water	1.19	0.042	10.5	0.020	1.83	0.49	0.34	0.075
	Supernatant	0.030	0.012	0.63	0.008	0.20	0.03	0.02	0.000
Ghosts washed in water	In buffer	0.110	0.002	0.15	0.002	0.04	0.28	0.28	0.051
	In water	0.230	0.001	0.15	0.003	0.05	0.30	0.32	0.073
	Supernatant	0.85	0.056	11.9	0.026	1.78	0.26	0.03	0.003

Table II. Total enzyme activities of the erythrocyte ghosts, resulting from the distribution of activities between membrane and supernatant fractions, as affected by the osmolarity of the wash media.

Sample	Total enzyme activity IU per ml.							
	GSSG-R	GSH-P	TPI	PK	LDH	PGK	TPDH	Aldolase
Control ghost suspension	1.15	0.059	11.6	0.027	1.94	0.54	0.40	0.075
Ghosts washed in 80 mOsm Tris buffer, pH 7.4	1.22	0.054	11.1	0.028	2.03	0.52	0.36	0.075
Ghosts washed in water	1.08	0.057	12.0	0.029	1.83	0.56	0.35	0.076

Total enzyme activity is the sum of the activity of membrane fraction assayed in water + activity of the supernatant fraction.

Table III. Retention of enzyme activities by the membrane fraction of the erythrocyte ghosts, as affected by the osmolarity of the wash media.

Enzyme	% activity retained by membrane fraction, after washing the ghosts in water.
GSSG - reductase	21 %
GSH - peroxidase	1.8 %
TPI	1.2 %
PK	10 %
LDH	27 %
PGK	54 %
TPDH	91 %
Aldolase	96 %

contact with the bulk of the liquid. If parts of the structural components of the membrane were enzymes (eg. aldolase and TPDH, i.e., the firmly bound enzymes), their catalytic sites could be freed from the interiors of the membrane, and would form complexes more readily with their substrates, resulting in a higher activity, than what they would give in the native state. As the ionic strength of the wash medium is increased, however, increasing proportion of these structural enzymes would remain in the native state, which would show up as a continuous decrease in the activity profile curves of these enzymes (Fig. 7). Phosphoglycerate kinase then could be partly structural protein, partly cytoplasmic enzyme in loose association with the erythrocyte ghost, since it bears the characteristics of both firmly and loosely bound groups of enzymes.

To further substantiate the structural role of the firmly bound enzymes, some means were sought by which an additional disintegration of the membrane could be achieved. It was believed that the further disintegration of the membrane would result in an additional increase in enzymic activity, over and above the cryptic activities obtained for the enzymes. Of the agents tested, the nonionic detergent Triton X-100, which is known to dissociate lipoprotein complexes of cell membranes, was found to be the most suitable to fulfill this requirement. At a concentration of 0.05 vol. % or over, TX-100 was found to decrease turbidity readings of the assay mixture at 660 nm from 0.2 - 0.3 absorbance initially to or close to zero. At concentrations used in the experiments, TX-100 was found to have no denaturing effect on the enzymes, which was apparent in each and every

Table IV. The effect of Triton X-100 concentration on enzyme activity.

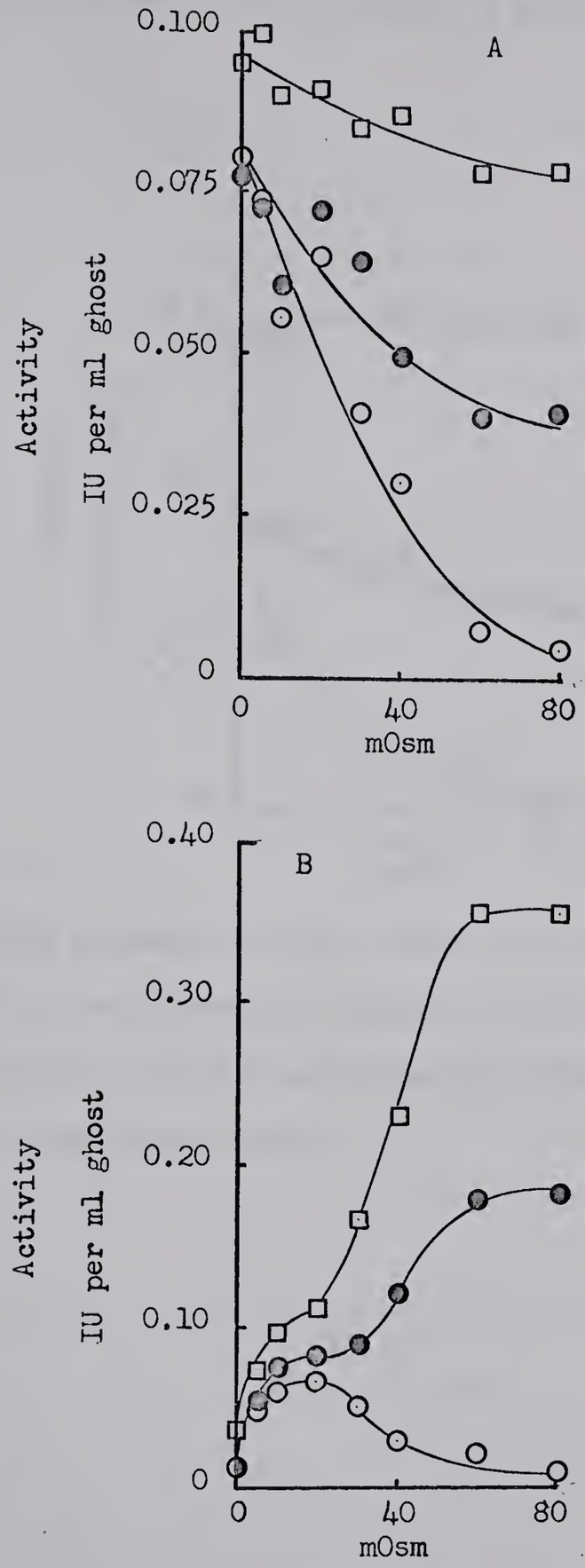
Concentration of TX-100 in the assay mixture (vol. %)	Activity IU per ml ghost	
	Aldolase	LDH
0	0.0050	0.145
0.006	0.029	1.05
0.012	0.110	1.15
0.025	0.108	1.15
0.050	0.105	1.15
0.10	0.110	1.15
0.20	0.106	1.16
0.50	0.109	1.16

Table V. The effect of Triton X-100 with time of incubation on the enzymic activities of the erytrocyte ghost.

Time of incubation in 0.15 vol. % TX-100 (mins)	Activity IU per ml ghost	
	Aldolase	LDH
1	0.115	1.15
2	0.117	1.14
4	0.114	1.12
8	0.114	1.13
20	0.117	1.14
40	0.115	1.14

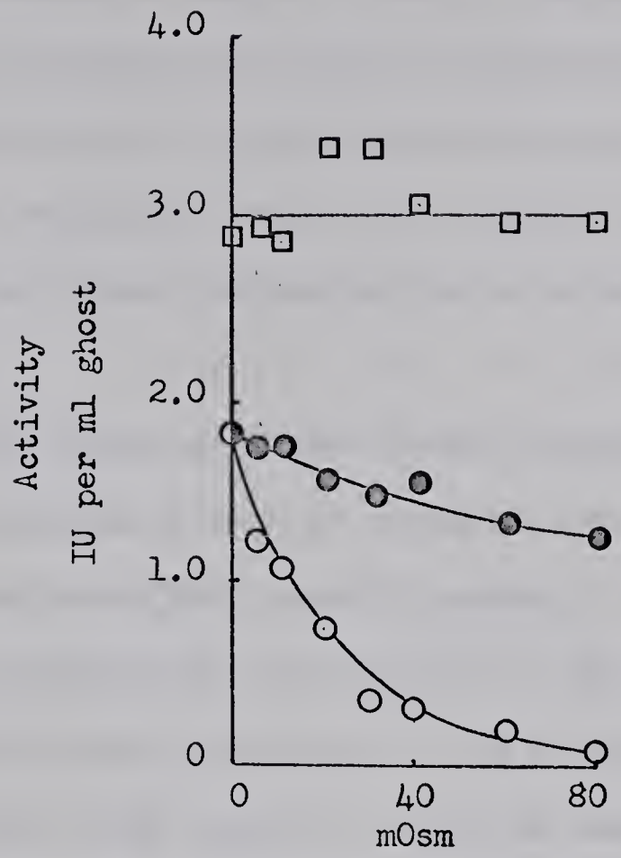
Ghosts were prepared in 10 mOsm MTT buffer, pH 7.4 in the presence of 400 mM Mg and Ca. In the experiment presented in table IV, aliquots of the ghost suspension were removed, to which varying concentrations of TX-100 were added prior to the enzyme assays. In the experiment presented in table V, the ghost suspension was made to contain 0.15 vol.% TX-100, of which aliquots were removed for enzyme assays at time intervals.

Fig. 8. Retention of enzyme activities by the erythrocyte ghosts.



Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. The ghosts were diluted six fold prior to the addition of the assay mixture (o) in the corresponding buffer; (●) in deionised water; (□) in water containing 0.05% Triton X-100. (A) aldolase; (B) phosphoglycerate kinase.

Fig. 9. Retention of TPDH activity by the erythrocyte ghosts.



Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. Prior to the assay of enzyme activity, the ghosts were diluted six-fold (o) in the corresponding buffer (•) in deionised water (□) in 0.05% Triton X-100.

case by the linearity of the progress curves. Some results of specific investigations aimed at uncovering any such effects of TX-100 are presented in tables IV and V. Table IV shows that, during the time course of the assay, concentrations of up to 0.4 vol.% of TX-100 has no effect on the enzyme activities, and Table V indicates that incubation of red cell membranes in 0.15 vol.% detergent up to 40 mins. is ineffective in altering the enzyme activities.

To test the effect of TX-100 on the activity profile curves of the enzymes, experiments were set up as described previously in connection with the activity profile studies. In addition, however, a third set of experiments was prepared, in which TX-100 was used as a diluent for the ghost suspensions. The concentration of TX-100 was 0.05 vol.% in the assay mixture. As can be seen in Figs. 8 & 9, the activity profile curves of the enzymes were further enhanced over their cryptic activity profiles, through the action of TX-100 on the erythrocyte ghosts. This enhancement of activity is in accord with the expectations, and provides further evidence on the possible structural role these enzymes may play in the erythrocyte membrane.

The effect of pH upon retention of enzymes by erythrocyte ghosts

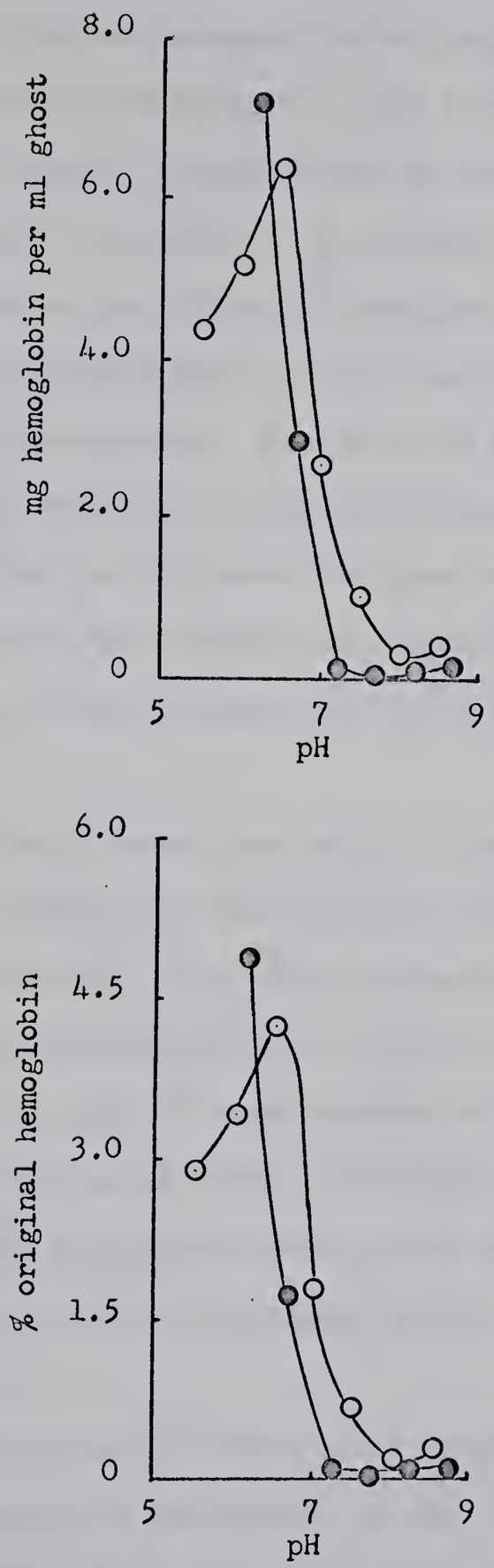
Studying the effect of pH on the hemoglobin content of the erythrocyte ghost, Dodge *et al.* (14) found substantial losses of non-hemoglobin nitrogen from the erythrocyte ghost, as the pH of the hemolyzing buffer was raised beyond 7.4. They suggested that the principal components were protein and/or mucoprotein in character.

Mitchell et al. (47) have confirmed the loss of non-hemoglobin protein form the erythrocyte ghosts at higher pH's, and attempted to characterize them. Although they were not able to show the nature of this non-hemoglobin protein, measurements of the activities of acetylcholinesterase (acetylcholine acetyl-hydrolase EC 3.1.1.7), aldolase, and TPDH in ghost prepared at different pH values indicated that none of these enzymes would be preferentially retained at lower pH's, and the loss of nonhemoglobin protein is therefore not associated with these enzymes.

Closer examination of these three enzymes on somewhat less conventional grounds reveals that they have one property in common, namely their close association with the erythrocyte membrane. While acetylcholinesterase of the erythrocyte is generally accepted to be localized in the membrane (69,70,71), aldolase along with TPDH is closely associated with it, on the basis of the osmolarity studies presented earlier. It is therefore not too surprising to find that these enzymes give only a slight response to changes in the pH of the hemolyzing buffer. But what about the loosely-bound enzymes? The experiments that follow are an attempt to answer this question.

One of the requirements in studying the effect of pH on the retention of enzymes by erythrocyte ghosts is to have a buffer with a wide range of buffering capacity. The ghosts were therefore prepared in MTT buffer, which fulfills this requirement. MTT is an equimolar mixture of MES, pK 6.15; TES, pK 7.5; and TRICINE, pK 8.15, and so it has an effective buffering range between pH 5.15 and pH 9.15.

Fig. 10. The effect of pH upon hemoglobin retention by erythrocyte ghosts



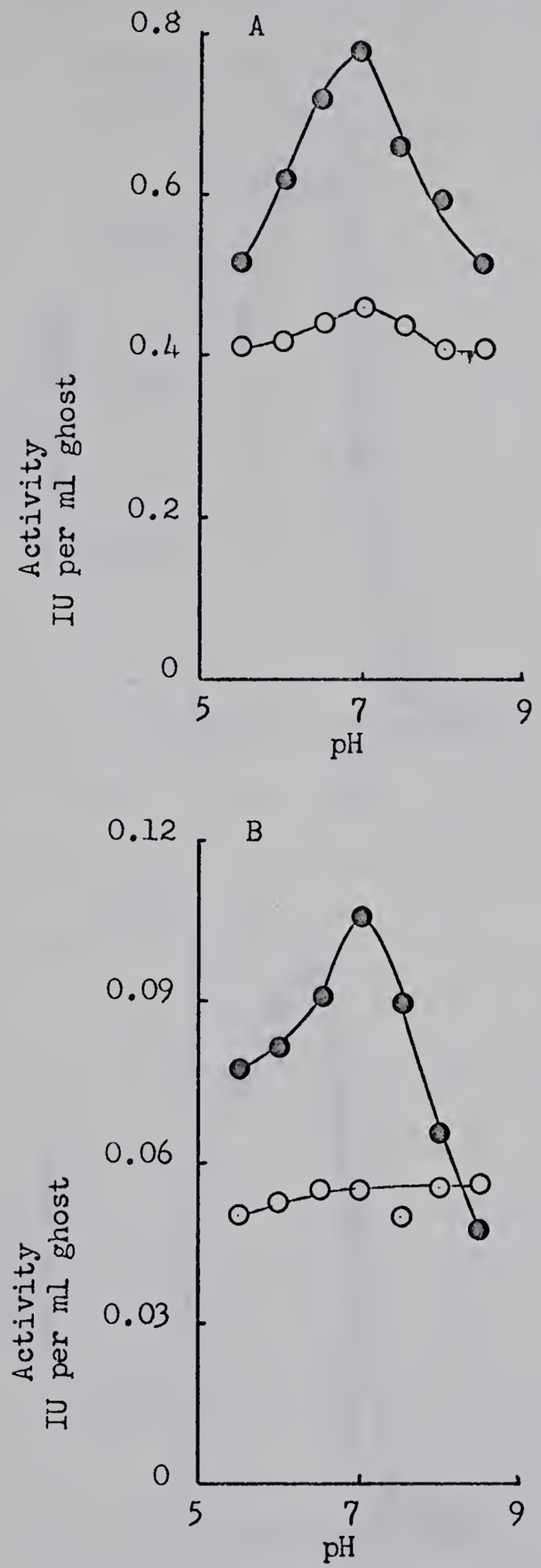
Ghosts were prepared in MTT buffer at the different pH values indicated. (o) 5 mOsm buffer, (•) 20 mOsm buffer.

Ghosts were prepared at different pH values as indicated in Figs. 10 - 14; however they were assayed for the enzymic activities at pH's given under "Materials and Methods". This is to state, that these studies were not aimed at investigating the effect of pH on enzyme kinetics, but rather to determine its influence on the retention of enzymic activities by the erythrocyte membrane. The small volume of ghost suspension added to the assay mixture was insufficient to change its pH significantly, even when the ghosts were prepared at the extremes of pH employed in these experiments. To assure free expression of enzymic activities of the ghost suspensions, prior to starting the reaction the aliquots were diluted six-fold in TX-100, the concentration of which yielded 0.03 vol.% in the final assay mixture.

The most striking visual observation in these experiments was the wide range of hemoglobin concentrations retained by the erythrocyte membrane at the different pH's. Some representative analytical data - which are in close agreement with the results of Mitchell *et al.* (47) - are presented in Fig. 10. A steep increase in hemoglobin content of the membrane can be ovserved in 5 mOsm or 20 mOsm buffer at the lower pH's. While at pH 7.7 the hemoglobin content of the ghost suspension is reduced to 0.05%, at pH 6.2 it retained 4.9% of the original RBC suspension.

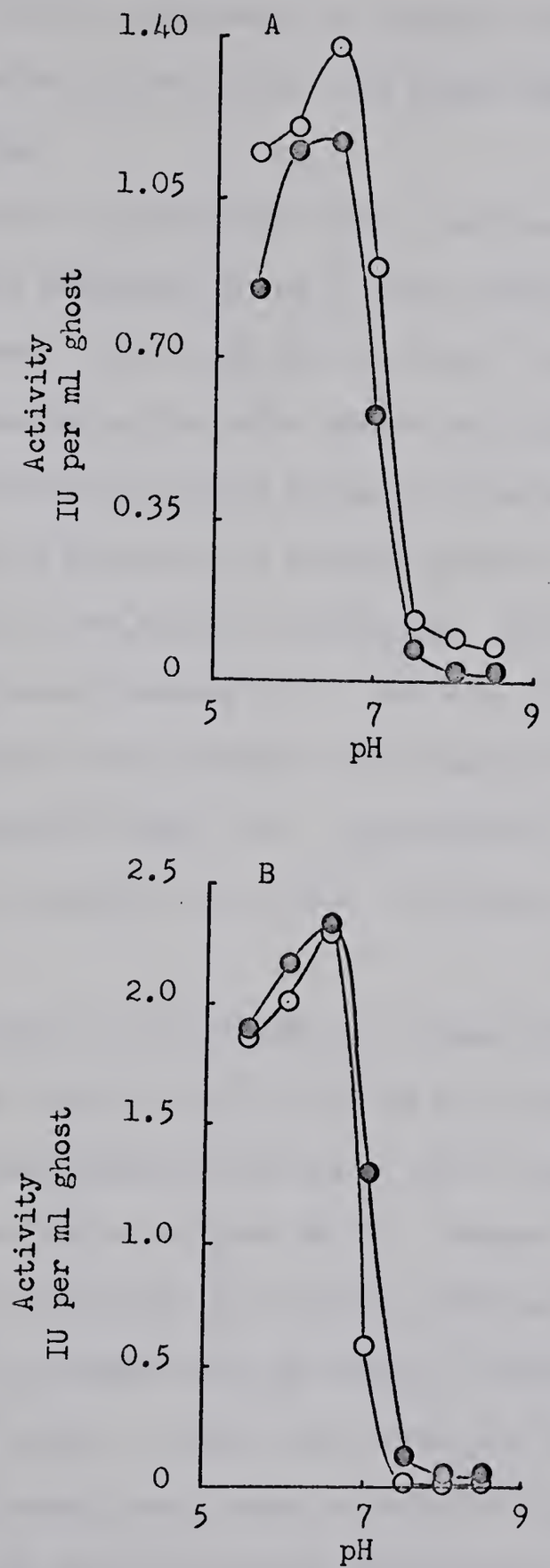
The effect of pH on two firmly bound enzymes (TPDH and aldolase) by the erythrocyte ghosts is represented in Fig. 11. These results are in close agreement with those of Mitchell *et al.* (47), who carried out the ghost preparation in 20 mOsm buffer, and found that these could not add

Fig. 11. The effect of pH upon enzyme retention by erythrocyte ghosts



Ghosts were prepared in MTT buffer, at the different pH values indicated. (o) 5 mOsm buffer, (•) 20 mOsm buffer. (A) TPDH, (B) aldolase.

Fig. 12. The effect of pH upon enzyme retention by erythrocyte ghosts



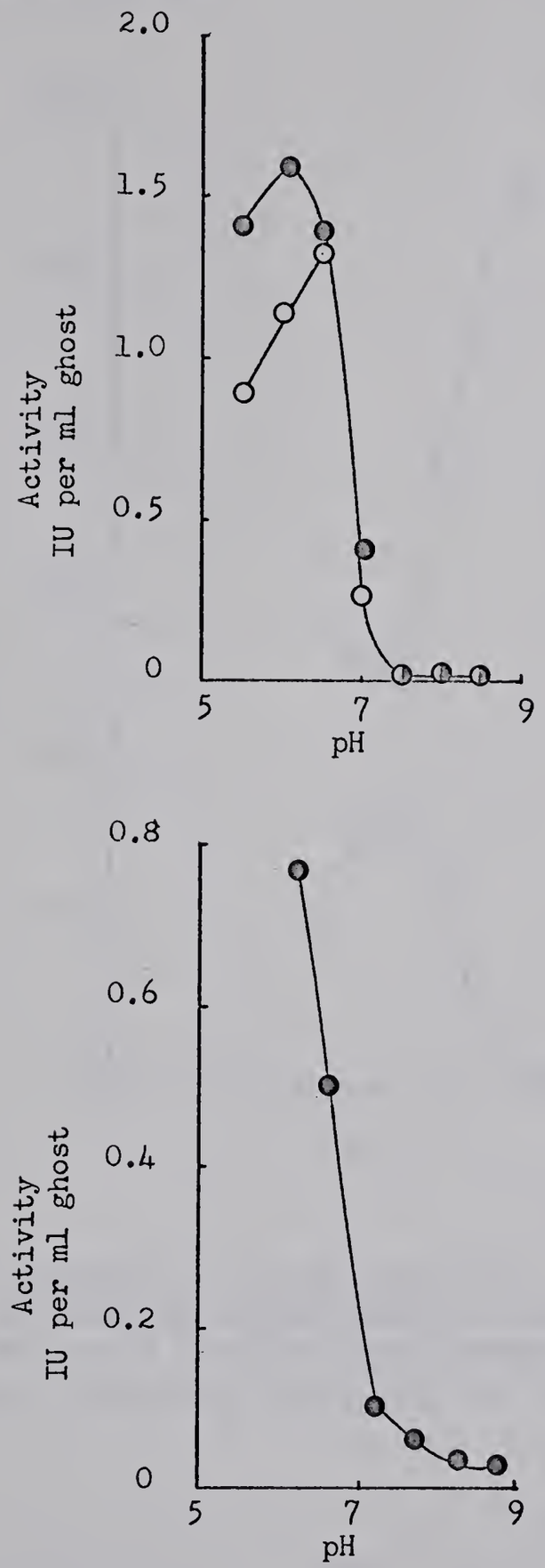
Ghosts were prepared in MTT buffer, at the different pH values indicated. (o) 5 mOsm buffer, (•) 20 mOsm buffer. (A) PK, (B) LDH.

significantly to the loss of non-hemoglobin proteins. It is to be noted, that when the preparation of ghosts is done in the 5 mOsm buffer, the effect of pH is even less significant upon the retention of these enzymes.

In contrast to aldolase and TPDH, the loosely-bound enzymes exhibit a large dependence on pH in their binding properties to the erythrocyte ghost. It can be seen in Figs. 12 and 13 that the removal of these enzymes is optimal when ghosts are prepared at or above pH 7.5. As the pH of the washing medium is lowered below this value, a sharp increase in retention of enzymic activity can be seen, which may be as high as two orders of magnitude. The maximal retention of these enzymes occurs between pH 6.0 and 6.5. It may also be observed that these loosely-bound enzymes show a pattern of retention similar to that of hemoglobin (Fig. 10). The osmolarity of the washing buffer seems to have no significant role in the pattern of retention of these enzymes.

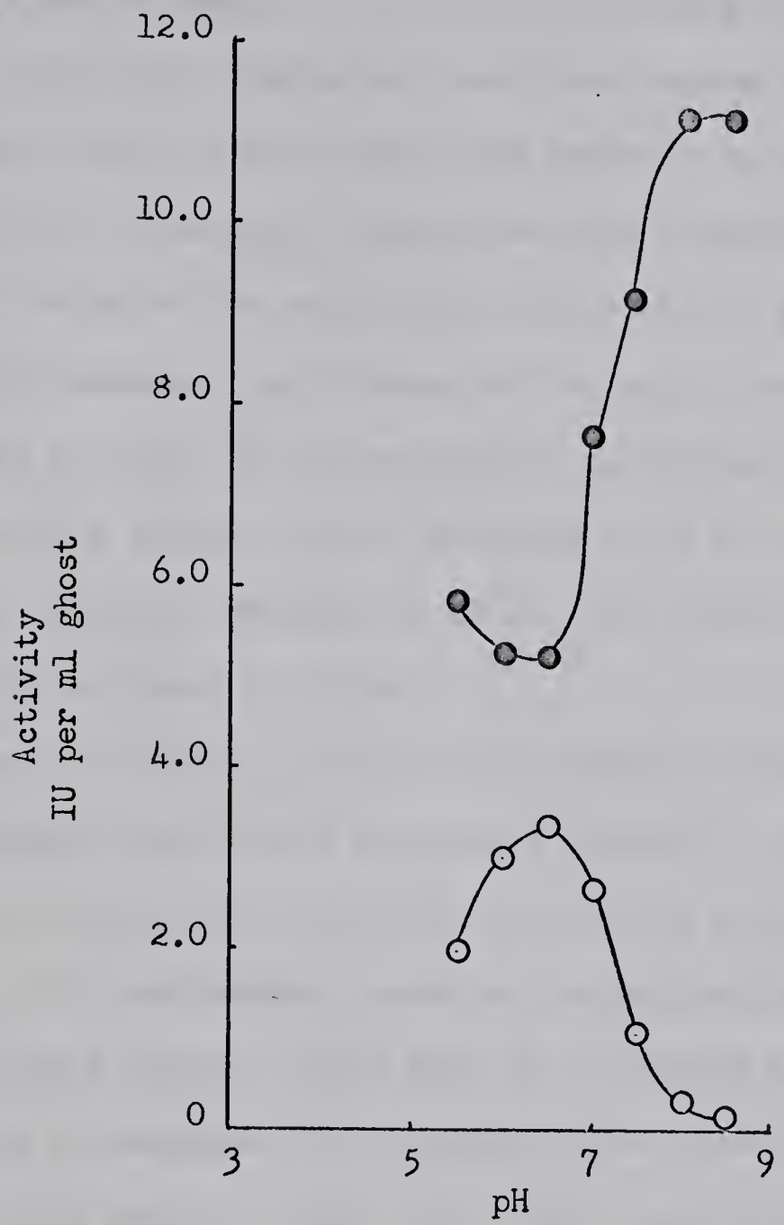
It was thought to be unlikely that these dramatic changes of activity were a direct result of the pH on the enzymes themselves in the form of denaturation at the higher pH's, since their optimal assay conditions are at or near pH 7.5. However it was decided to eliminate this possibility by titrating and backtitrating the ghost suspensions to the appropriate pH values, followed by assaying for the particular enzyme. It has been found that this change in pH resulted in no significant change of enzymic activity. A further check on this point was obtained through assaying for enzymic activity in the first supernatant fraction obtained during the preparation of ghost

Fig. 13. The effect of pH upon enzyme retention by erythrocyte ghosts



Ghosts were prepared in MTT buffer at the different pH values indicated. (o) 5 mOsm buffer, (•) 20 mOsm buffer. (A) GSH-P, (B) PGK.

Fig. 14. The effect of pH of the hemolysing buffer, on the distribution of LDH activity, between the membrane fraction and stroma free hemolysate of the erytrocyte.



Ghosts were prepared in 30 mOsm MTT buffer, at the different pH values indicated. LDH activity was determined in the first supernatant liquid (●), as well as in the final ghost suspension (o).

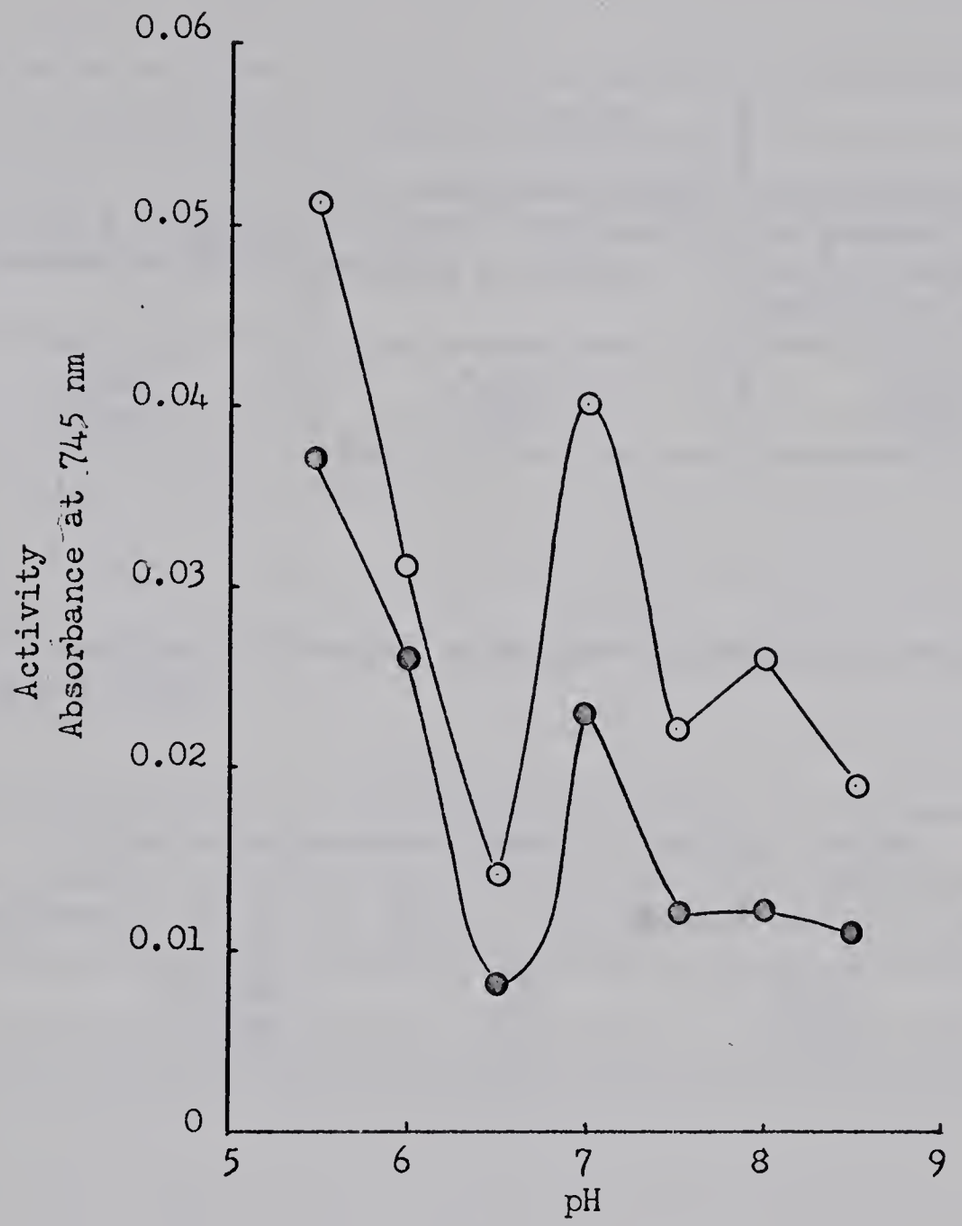
* Total hemolysate activity in the presence of Triton X-100 is 13.7 IU per ml.

suspensions. Such a result is presented for LDH in Fig. 14. It can be seen that the activity of the enzyme in the supernatant fraction is actually a mirror image of the activity obtained by the ghosts at the different pH values, indicating that these enzymes are indeed specifically bound to the erythrocyte membrane at the lower pH's.

ATP-ase, a 'membrane' enzyme, was also investigated in regard to the effect of pH of the preparatory buffer on its activity in the erythrocyte membrane. An "irregular" but highly reproducible pattern obtained as a result of this experiment is presented in Fig. 15. There appears to be a smaller peak of activity at pH 8.0 and a larger one at pH 7.0. A low point obtained at pH 6.5 is followed by an ascending arm towards the lower pH values.

It was of further interest to see whether the pH of the wash medium has a permanent effect upon the enzyme content of the erythrocyte ghosts once they are prepared, or whether it could be altered through adjustment of its pH. To test whether there is a recombination of the enzymes with the erythrocyte ghosts, 5 mOsm MTT, pH 7.4 buffer was employed in the preparation of membranes. The membrane-free supernatant of the first hemolysate was saved for later use. After completion of the preparation, the ghosts were divided in three parts. Aliquot (A) was transferred to a centrifuge tube and was washed three more times in the pH 7.4 buffer. Aliquot (B) was washed once with the membrane-free hemolysate followed by two more washings in the pH 7.5 buffer. Aliquot (C) was washed once in the membrane-free hemolysate, but the pH of the suspension was adjusted to 6.5 prior to centrifugation, which was followed by two more washes in pH 6.5 buffer. It may be seen in Table VI

Fig. 15. The effect of pH upon retention of ATP-ase activity by the erythrocyte ghosts.



Ghosts were prepared in 5 mOsm MTT buffer at the different pH values indicated. (o) total ATP-ase activity, (●) ouabain-insensitive ATP-ase activity. (the assay mixture contained 10^{-4} M ouabain).

Table VI. The effect of changes in pH upon binding of enzymes to the erythrocyte ghosts.

Enzyme assayed	IU per ml ghost		
	(A) Ghosts prepared and washed at pH 7.5	(B) Hemolysate added to ghosts followed by washing at pH 7.4	(C) Hemolysate added to ghosts, pH adjusted to 6.5 followed by washing at pH 6.5
PK	0.110	0.113	0.40
LDH	0.310	0.320	0.83

Table VII. The effect of changes in pH upon liberation of enzymes from the erythrocyte ghosts.

Enzyme assayed	IU per ml supernatant based on original volume	
	supernatant of pH 6.5 ghosts	Supernatant of pH 7.4 ghosts
PK	0.080	0.85
LDH	0.220	2.54

that ghosts prepared in pH 7.5 buffer have recombined with the enzymes of the membrane-free hemolysate, when the pH was changed to the lower value, and even repeated washing of the membrane with pH 6.5 buffer failed to remove the enzymes. No recombination of the enzymes occurred, however, when the pH was kept at 7.5.

To test whether the enzymic activity can be liberated from the ghosts prepared at pH 6.5 by change in the pH, an aliquot of ghosts was diluted ten-fold in the same 5 mOsm MTT, pH 6.5 buffer, in which the preparation was carried out. Another aliquot of the ghost suspension was titrated to pH 7.5 and was subsequently diluted in a 5 mOsm MTT, pH 7.5 buffer. After centrifugation the supernatants were tested for PK and LDH activity. A significant increase in enzymic activity may be seen in the supernatant of the ghosts which has been titrated to pH 7.5 (Table VII), over the supernatant of the control ghost suspension, indicating that these enzymes may be easily removed from the membrane, by a simple change in pH of its suspension.

Firmly-bound enzymes on the other hand shown neither liberation from, nor recombination with, the erythrocyte ghosts under similar conditions.

The effect of Ca and Mg on retention of enzymes and hemoglobin by erythrocyte ghosts

Membrane Mg and Ca have received little attention in ultra-structural research, even though these ions have long been implicated in playing some role in the maintenance of erythrocyte integrity, in producing profound changes in membrane permeability. In the early

osmotic fragility studies of Brinkman (69), Ca was shown to suppress the initial hemolysis of the erythrocyte. The red blood cells of the tortoise and snapping turtle were shown to hemolyze in isotonic NaCl solution, unless small amounts of Ca were present (70,71). Other investigations have revealed a connection between Mg or Ca and membrane permeability in relation to univalent cations. While erythrocyte suspended in a nonelectrolyte such as sucrose in the presence of Mg and/or Ca show normal permeability (71), the absence of these ions renders the membrane abnormally permeable to Na and K. Once the cells are exposed to a solution of non-electrolytes, subsequent addition of Ca (not Mg) restores normal membrane permeability. Hoffman (72) calls Mg an internal membrane cohesive, which controls univalent cation permeability through stabilization of the internal molecular arrangement of the membrane. He found that erythrocyte ghosts reconstituted in the absence of Mg did not retain K ions as well as cells reconstituted in the presence of these ions. Schrier (6) observed that erythrocyte membranes prepared in the absence of Mg, when "dissected" with sonic oscillation or lipid-active agents, underwent an increase in activity of PGK but not of TPDH. When ghosts were prepared in the presence of Mg, however, an increase in activity of both PGK and TPDH was apparent on dissection.

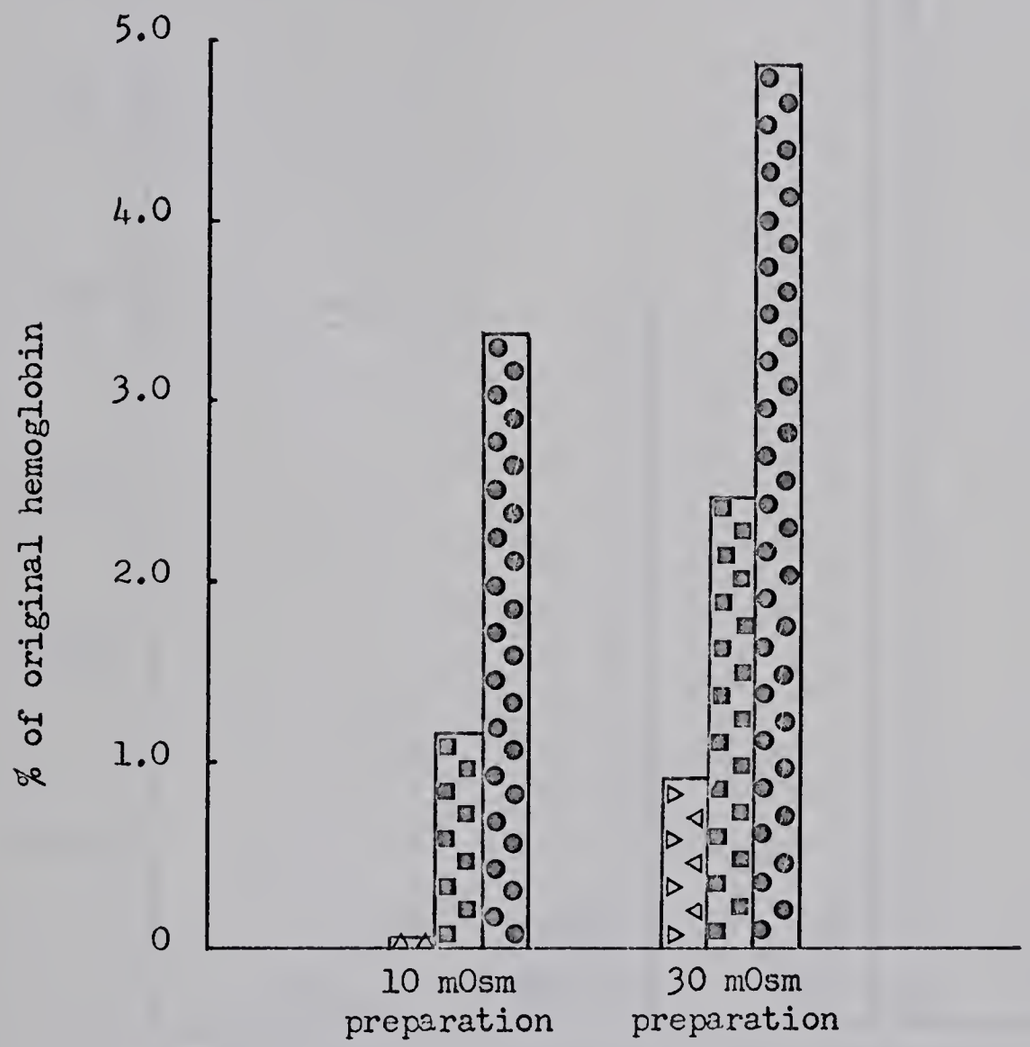
The experiments that follow attempts to investigate the effect of Mg and Ca on hemoglobin and enzyme retention by the erythrocyte ghosts, as well as to study these ions and their effect in relation to membrane integrity. Ghost suspensions prepared in the presence of these ions will be referred to as "Mg-ghosts" and "Ca-ghosts", respectively, while

the membrane preparations carried out in the absence of these ions will be called "standard ghosts".

To test the effect of Mg and Ca upon retention of hemoglobin by the erythrocyte ghost, red cell suspensions were hemolyzed and subsequently washed four times in 10 mOsm and 30 mOsm Tris buffer, pH 7.4. One of the samples in each preparation was made to contain 0.5 mM of either Mg or Ca. It may be seen in Fig. 16 that standard ghosts prepared in the 30 mOsm buffer retained substantially more hemoglobin than ghosts prepared in the 10 mOsm buffer, which finding confirms the report of Dodge, et al. (14). An even more dramatic change in hemoglobin content of the ghosts resulted from the presence of Mg or Ca in the washing buffer. The increase in hemoglobin content of the ghosts due to the presence of Mg and Ca is expressed in both the 10 mOsm and the 30 mOsm preparations. Of the two ions Ca appears to be much more potent than Mg. It may be seen, however that although the absolute value of hemoglobin concentration is higher in the 30 mOsm preparation, the ratio of the hemoglobin content in the Mg-ghosts and Ca-ghosts to that in the standard ghosts is lower than that of the 10 mOsm preparation. While in the 10 mOsm preparation the ratios are 14 for Mg-ghosts and 70 for the Ca-ghosts, in the 30 mOsm preparation these ratios are reduced to 2.5 and 5, respectively.

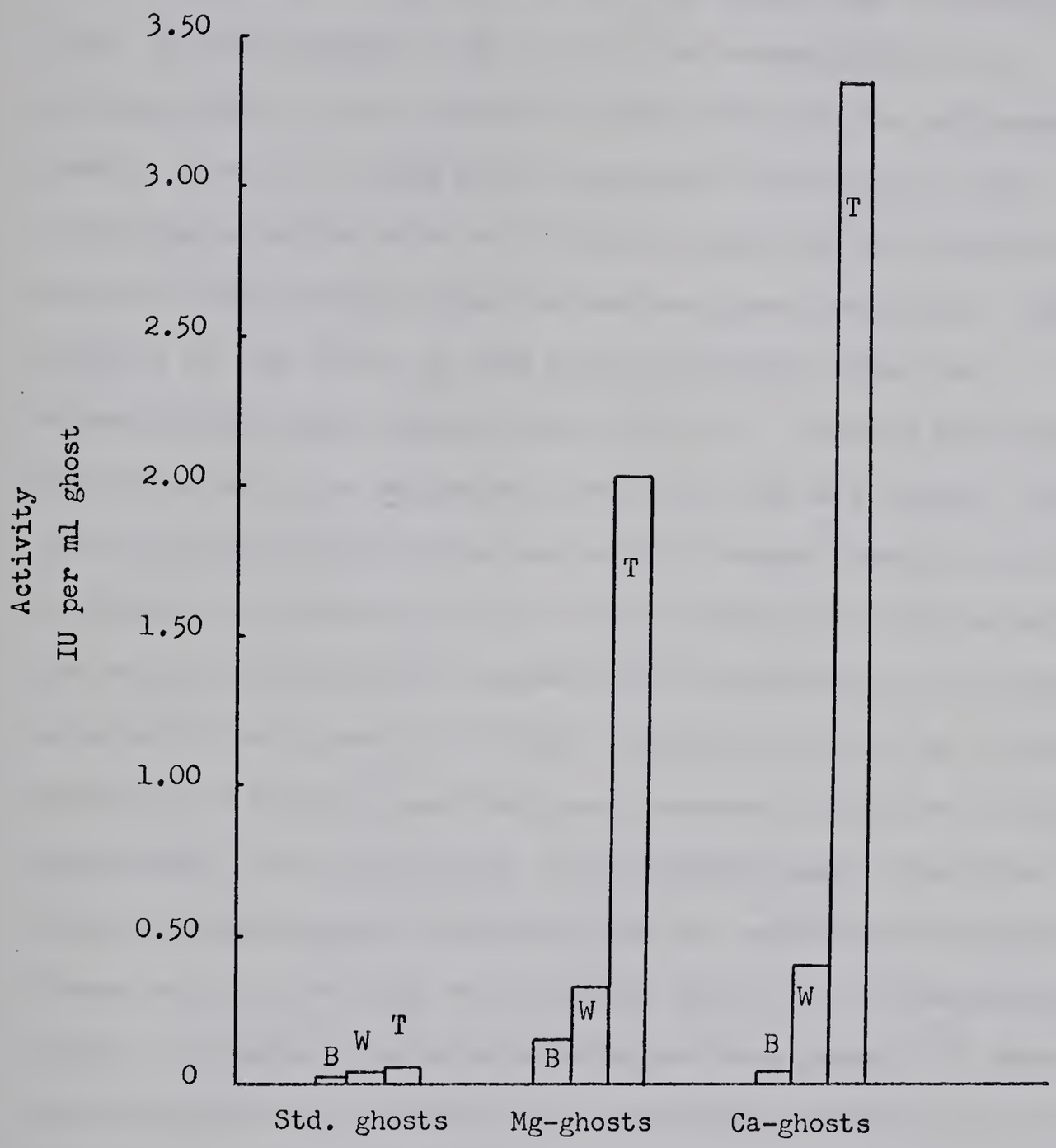
Figs. 17 & 18 indicate the effect of Mg or Ca ions on retention of LDH activity by the erythrocyte ghosts, under three sets of conditions. Ghost suspensions - prepared in 10 mOsm and 30 mOsm Tris buffer, pH 7.4, in the presence and absence of 0.50 mM of either Mg or Ca - were diluted six-fold in the corresponding buffer, in

Fig. 16. The effect of Mg or Ca on hemoglobin retention by erythrocyte ghosts.



Ghosts were prepared in Tris buffer, pH 7.4 at the osmolarities indicated. (△) standard ghosts, (■) Mg-ghosts, (●) Ca-ghosts.

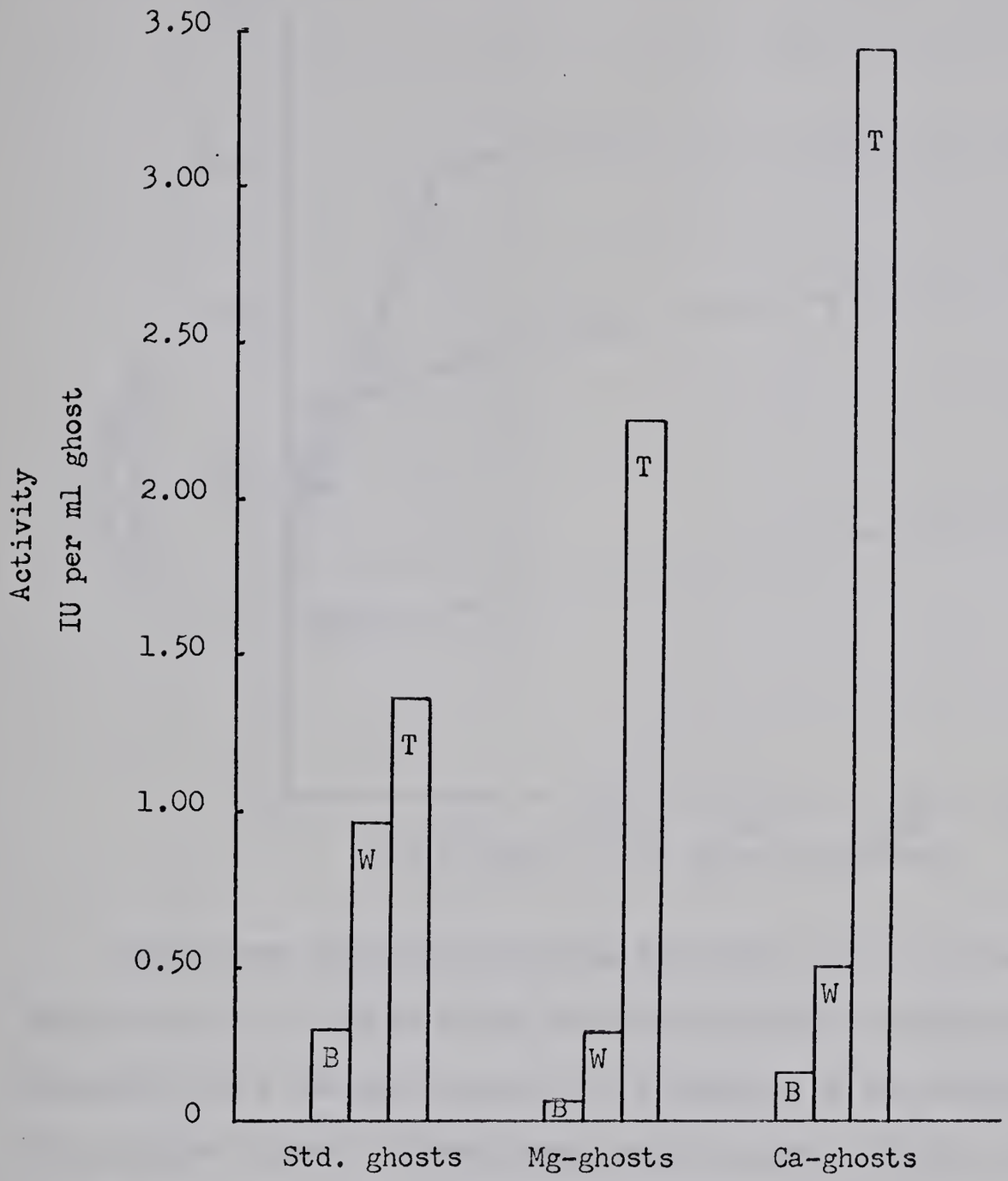
Fig. 17. The effect of Mg or Ca on LDH retention by erythrocyte ghosts



Ghosts were prepared in 10 mOsm Tris buffer, pH 7.4 in the presence and absence of 0.5 mM of either Mg or Ca. Prior to assay, they were diluted six-fold in (B) Corresponding buffer, (W) Deionised water, (T) 0.1 vol.% TX-100

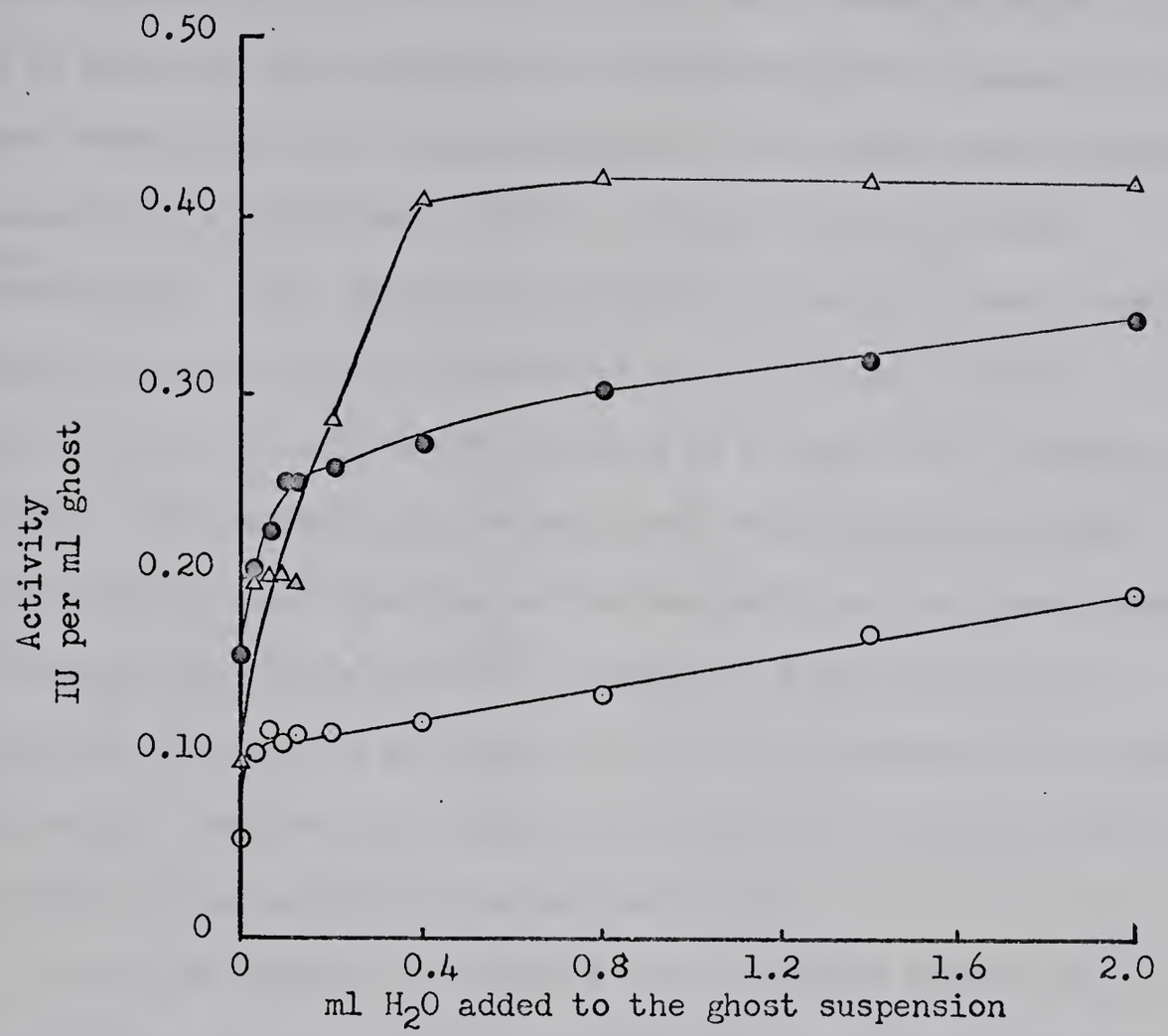
deionized water, or in 0.1 vol.% TX-100. It may be seen that the activity pattern obtained by the standard ghost preparations are in accord with data presented previously. As in the case of hemoglobin (Fig. 16), the presence of Mg or Ca in the washing buffer has a profound effect on the retention of LDH activity by the erythrocyte ghosts. When the 10 mOsm ghost suspensions were diluted in the corresponding buffer prior to the enzymic assay, Mg and Ca-ghosts had slightly higher activity than the standard ghost preparation. The opposite is true in the 30 mOsm ghost preparation, where the standard ghosts show somewhat higher activity. Although the overall activities more than doubled when water was used as a diluent, the activity pattern remained the same as that obtained through the use of buffer. The effect of Mg or Ca on the retention of LDH activity by the erythrocyte ghosts is revealed only on comparison of activities obtained in the presence of TX-100. As may be seen in Fig. 17, LDH activities of Mg-ghosts and Ca-ghosts increased 40-fold and 75-fold respectively, over the activity of the standard ghost preparation. So this enhanced enzyme retention of the Mg- and Ca-ghosts remains a "latent activity" as long as the diluent used is the corresponding buffer. It begins to be revealed when the ionic strength of these ghost suspensions is reduced prior to the assay procedure for LDH, and it culminates when TX-100 is present in the reaction mixture. "Latent activity" therefore will refer to the enzymic activities that, although present in the Mg- and Ca-ghosts, can not be measured due to the circumstances of the assay conditions.

Fig. 18. The effect of Mg or Ca on LDH retention by erythrocyte ghosts



Ghosts were prepared in 30 mOsm Tris buffer, pH 7.4 in the presence and absence of 0.50 mM of either Mg or Ca. Preor to assay a six-fold dilution was obtained in (B) Corresponding buffer, (W) Deionised water, (T) 0.1 vol.% TX-100

Fig. 19. The effect of lowering the ionic strenght of the suspending medium upon the LDH activity of standard, Mg & Ca-ghosts.



Ghosts were prepared in 30 mOsm MTT buffer, pH 7.4 in the presence and absence of 1.0 mM Ca or Mg. To 0.10 ml ghost increasing volumes of deionised water was added prior to the addition of the assay mixture. (△) standard ghosts, (o) Mg-ghosts, (●) Ca-ghosts. (In the presence of TX-100, activities were increased to: 0.45 IU per ml standard ghost; 0.70 IU per ml Mg-ghost; and 1.00 IU per ml Ca-ghost.

Fig. 19 indicates the LDH activity of standard, magnesium, and calcium ghosts as a function of dilution with deionized water. It may be seen that the activity of the standard ghost preparation has a first-order relation to the osmolarity of the medium, which rapidly changes into a zero-order relation of around six mOsm buffer concentration. Even though LDH activity leveled off, once a six-fold reduction in osmolarity of the ghost suspension was reached, dilution in 0.10 vol.% TX-100 resulted in a slight (6%) increase in activity. LDH activity in the Mg-ghosts and Ca-ghosts increased rapidly upon slight reduction of the osmolarity of the ghost suspension on addition of deionized water, followed by a much slower but continuous increase in activity up to 20-fold dilution of the suspension with water. Even at this high rate of dilution, their activity fell far short of the activity obtained in TX-100.

It was of interest to search for any possible pattern of regularities by comparing the retention of some of the loosely-bound and firmly-bound enzymes by standard, Mg-, and Ca-ghosts. The ghost preparations were carried out in 10 mOsm Tris buffer, pH 7.4 in the absence and the presence of 1.0 mM of either Mg or Ca. The results obtained for the four enzymes are presented in Table VIII.

According to expectations from previous data, the activities of the enzymes were the highest when they were diluted in TX-100, while the lowest activities were obtained when buffer was used as a diluent. Comparing the activities of the individual enzymes of the standard ghost preparations, the firmly-bound enzymes (TPDH, aldolase) show little variation, while the activities of the loosely-bound

Table VIII. The effect of Mg and Ca ions upon enzyme retention by erythrocyte ghosts

Enzyme assay	Sample	IU per 10^{10} ghost		
		Ghosts diluted in buffer	Ghosts diluted in water	Ghosts diluted in TX-100
PK	Std. ghost	0.022	0.027	0.040
	Mg-ghost	0.018	0.045	0.180
	Ca-ghost	0.038	0.062	0.35
LDH	Std. ghost	0.0130	0.030	0.045
	Mg-ghost	0.45	0.78	3.9
	Ca-ghost	0.37	1.89	6.3
Aldolase	Std. ghost	0.142	0.142	0.165
	Mg-ghost	0.047	0.069	0.158
	Ca-ghost	0.036	0.058	0.160
TPDH	Std. ghost	0.52	0.52	0.52
	Mg-ghost	0.100	0.175	0.53
	Ca-ghost	0.021	0.021	0.53

Ghosts were prepared in 10 mOsm Tris buffer, pH 7.4

Concentrations of Mg and Ca were 1.0 mM

enzymes (PK,LDH) show a slight enhancement, as the diluent is changed from buffer to deionized water to TX-100. Making the same comparison on Mg- and Ca-ghosts, it may be seen that both the loosely-bound enzymes and the firmly-bound enzymes show enhancement of activity in the direction just mentioned. Apart from the minor difference mentioned above, there are two major differences between the loosely-bound and firmly-bound enzymes of Table VIII. One of these differences is that, while latent-activity of the loosely-bound enzymes is higher in Mg- and Ca-ghosts than the activity of the standard ghosts (Ca-ghosts being the highest), the firmly-bound enzymes show decreasing latent-activity in the presence of the ions, with Ca-ghosts giving the lowest activity. The second important difference between the two groups of enzymes is that, while the retention of the loosely-bound enzymes - as revealed in the presence of TX-100 - is markedly affected by the presence of Mg or Ca in the washing buffer, the retention of the firmly-bound enzymes is completely unaffected by the presence of these ions.

On the basis of the results presented in Figs. 16-19, it was decided to do some morphological studies on the different ghost preparations, that might lead to an understanding of these results. The ghost preparations were carried out in 10 m0xm and 30 m0sm MTT buffers, pH 7.4, in the absence and the presence of 0.5 mM of either Mg or Ca. Prior to microscopic examination of the ghost suspensions, they were diluted six-fold either in the buffer in which they were prepared, or in deionized water. The photomicrographs are presented in Plates I-IV. It may be seen in Plates I-A, II-A, & II-C that

ghosts prepared and viewed in the 30 mOsm buffers have an "intact" appearance, regardless of whether Mg or Ca was present in the wash medium. A six-fold dilution of these ghost preparations in water however leaves the Mg- and Ca-ghosts appearing somewhat swollen but intact (Plates II-A & C). Standard ghosts, on the other hand, appear to be very faint, either highly swollen, fragmented, or having long ribbon-like or tubular forms attached to bulbar heads (Plate I-B). Formation of small vesicles on addition of NaCl to this ghost preparation is apparent in Plate I-C.

Plates III and IV are photomicrographs of the 10 mOsm ghost preparations. Plates III-A & B reveal that ghosts are highly fragmented when the osmolarity of the wash liquid is lowered to 10 mOsm. The presence of Mg or Ca ions in the wash medium however exerts a protective action against fragmentation of the ghosts, when the osmolarity of the suspending medium is reduced to 10 mOsm or less (Plates III-C & D, IV-A & B).

On comparison of the general appearance of the ghost preparations, one may observe that ghosts prepared in the presence of Mg are spherical, without any indentation, and have a cactus-like, prickly appearance. Calcium ghosts on the other hand are smooth, biconcave, or cup-shaped, and more closely resemble the standard ghost preparation.

It was shown that Mg and Ca protect the erythrocyte ghosts against hypotonic disintegration (Plates III and IV). It was therefore of interest to determine what concentrations of the ions are required for this protective action. Figs. 20 & 21 depict the effect of Mg or

Plate I. Ghost preparation was carried out in 30 mOsm MTT buffer, pH 7.4. Prior to microscopic examination, the ghosts were diluted six-fold (A) In the same buffer, (B) In deionized water, (C) In deionized water, but after 10 mins. of incubation at room temperature, the suspension was made isotonic with NaCl solution. Magnification 1000 X.

Plate I.

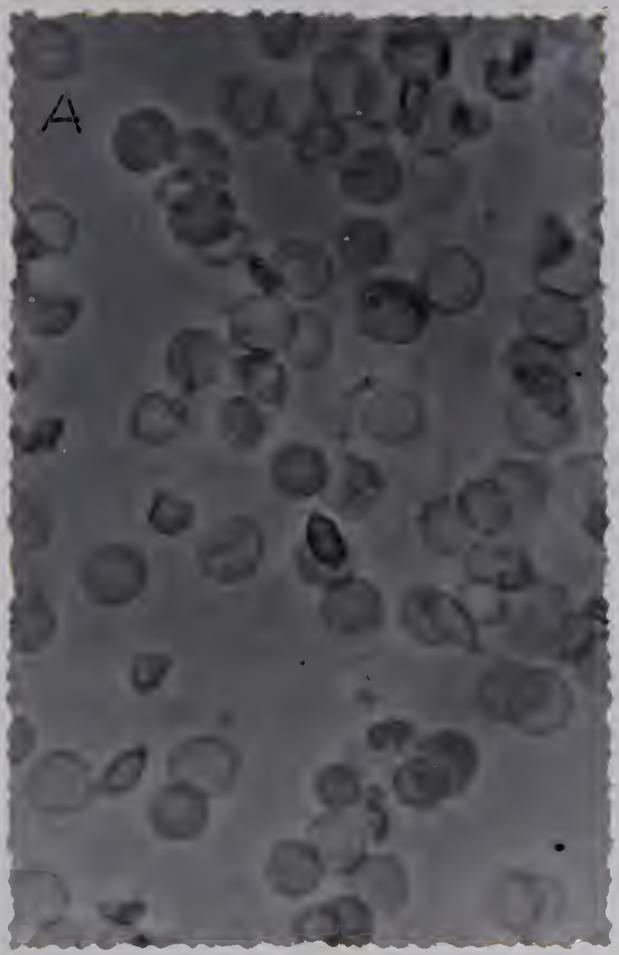


Plate II. Ghost preparation was carried out in 30 mOsm MTT buffer, pH 7.4, in the presence of 0.5 mM of either Mg or Ca. They were subsequently diluted six-fold either in the corresponding buffer, or in deionized water. (A) Mg-ghosts in the medium, (B) Mg-ghosts in deionized water, (C) Ca-ghosts in the medium, (D) Ca-ghosts in deionized water.

Magnification 1000 X.

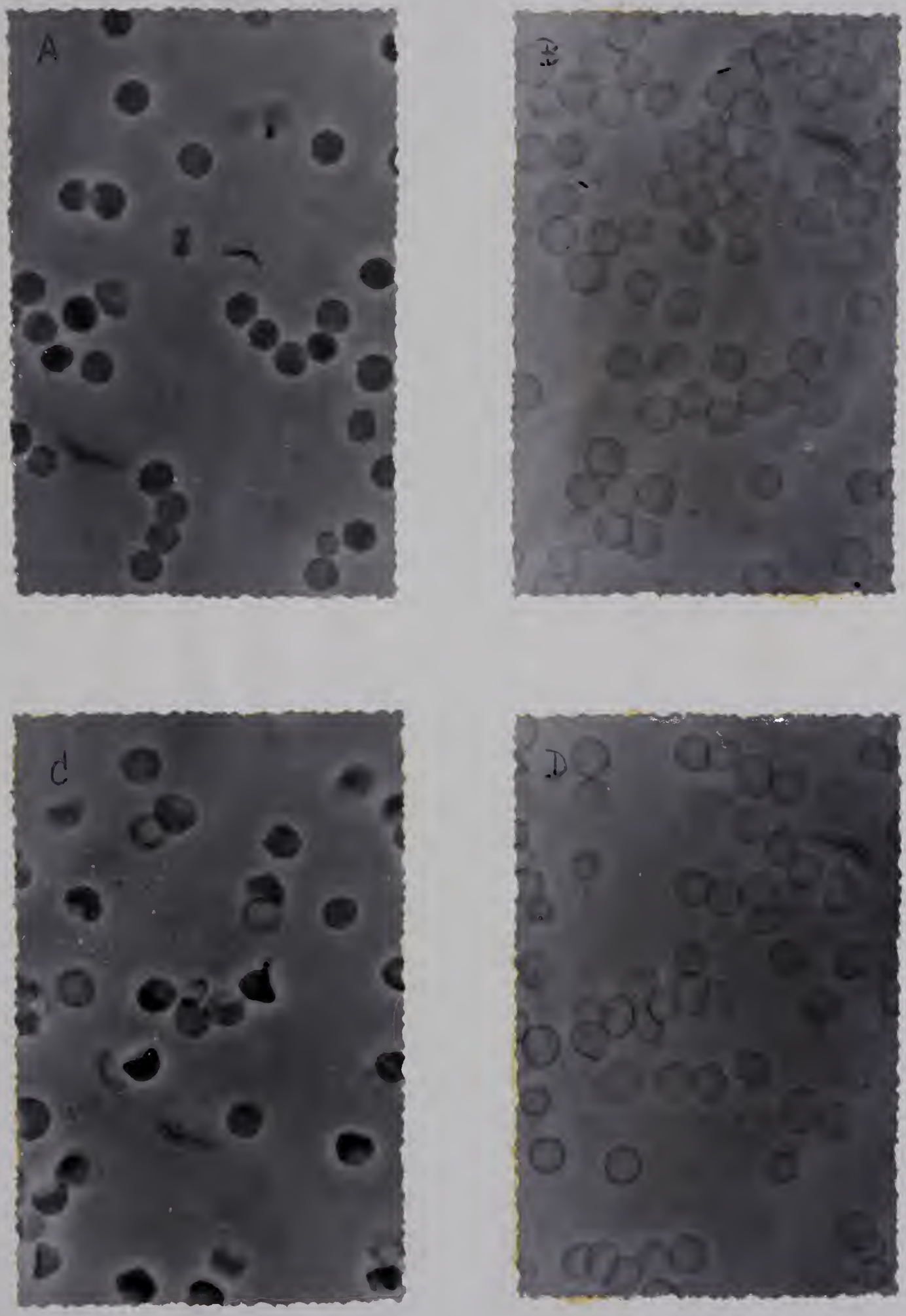
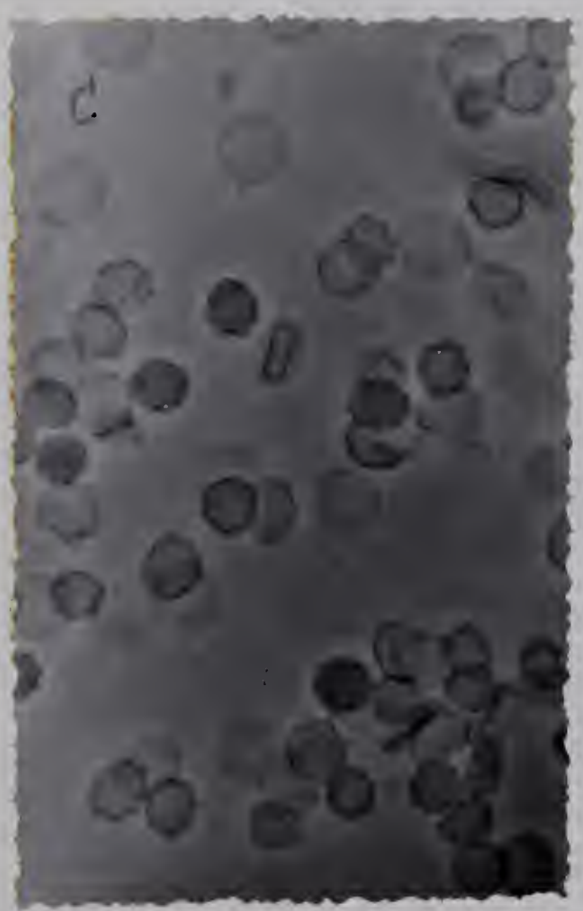

A
B
C
D

Plate III. Ghosts were prepared in 10 mOsm MTT buffer, pH 7.4 in the absence and presence of 0.5 mM Mg. The subsequent six-fold dilutions were done either in the wash medium or in deionized water. (A) Standard ghosts in the medium, (B) Standard ghosts in water, (C) Mg-ghosts in the medium, (D) Mg-ghosts in water. Magnification 1000 X.



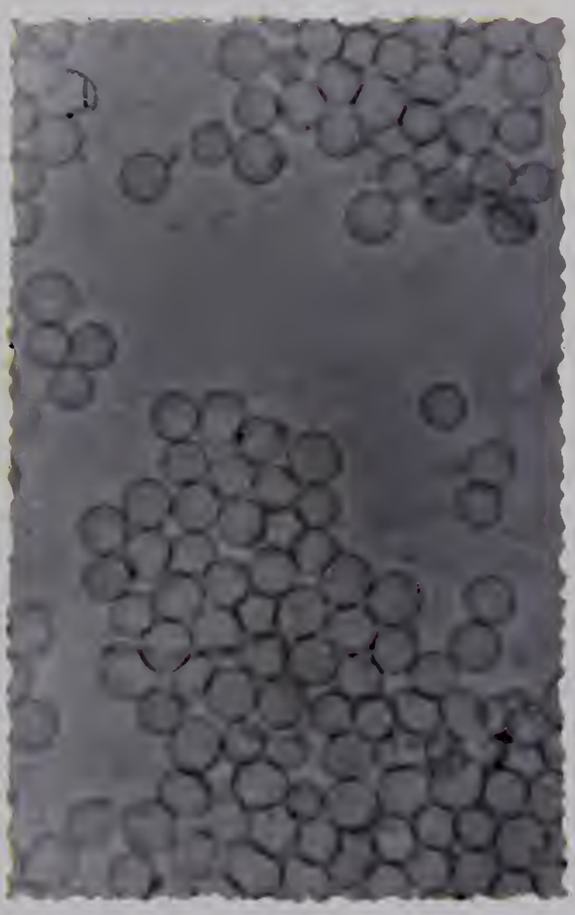


Plate IV. Ghosts were prepared in 10 mOsm MTT buffer, pH 7.4, in the presence of 0.5 mM of Ca. They were subsequently diluted six-fold in (A) Wash medium, (B) Deionized water.

Magnification 1000 X.


A

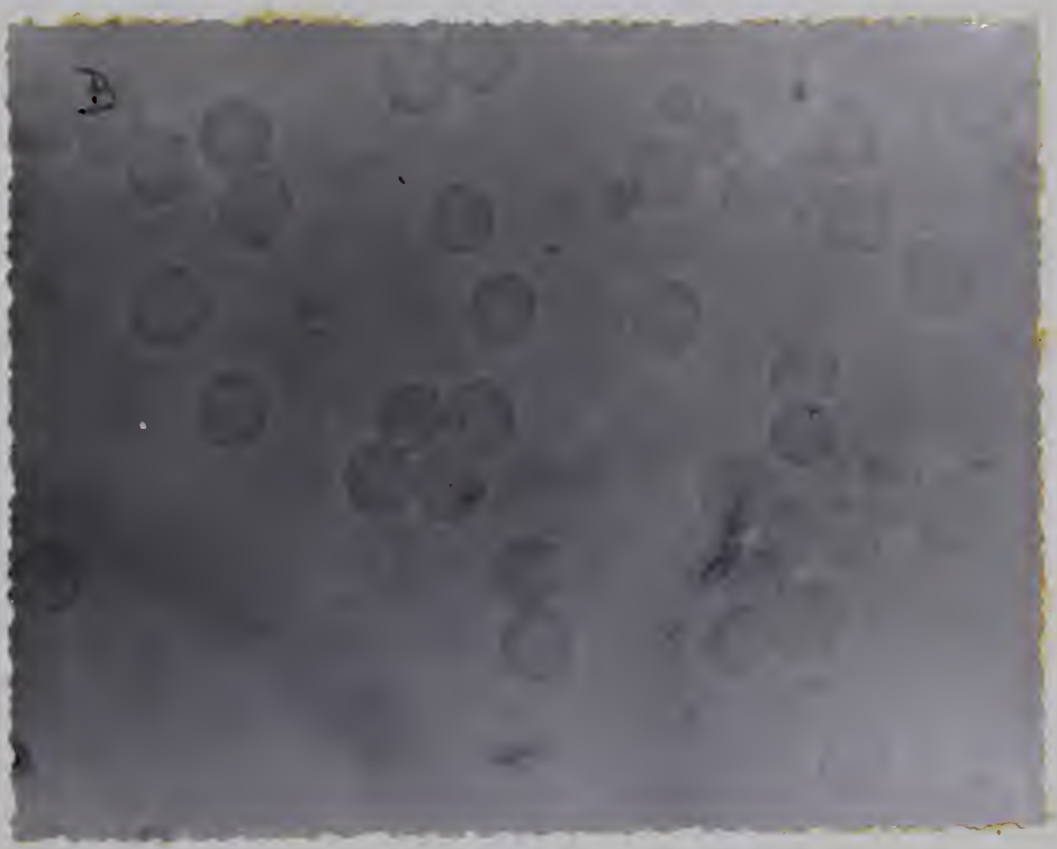
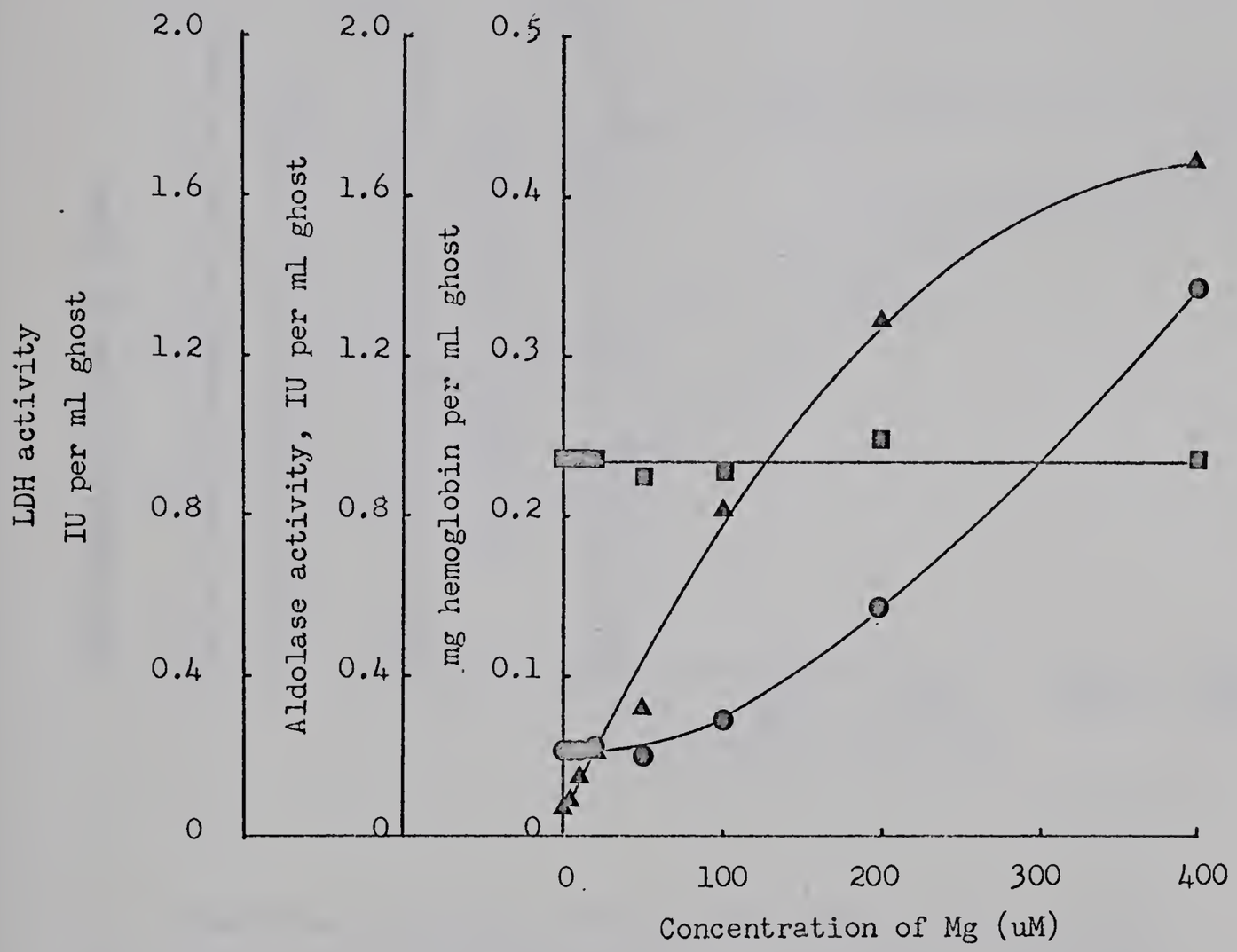
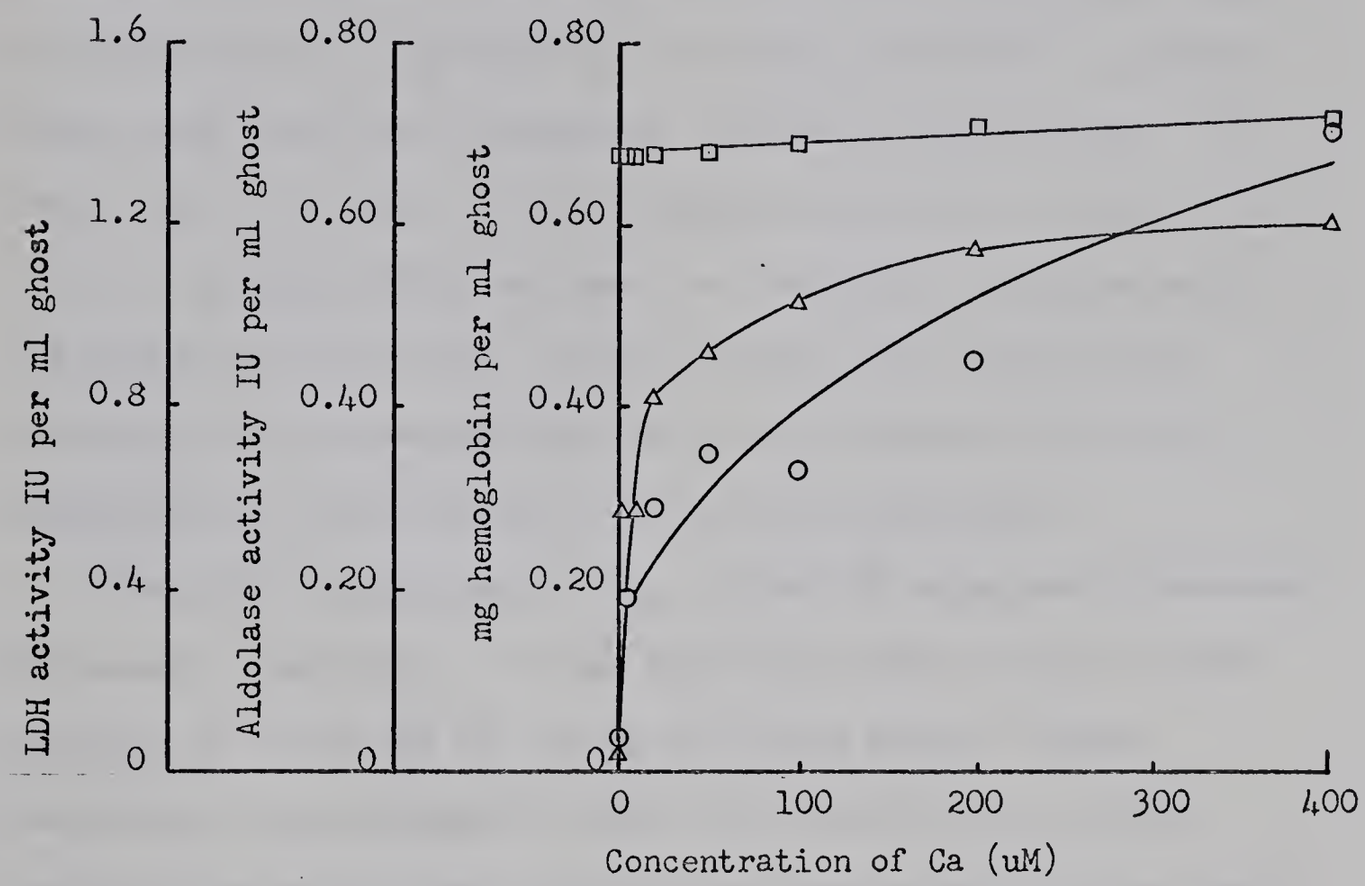

B

Fig. 20. The effect of Mg concentration in the washing buffer upon retention of hemoglobin, LDH and aldolase, by the erythrocyte ghosts



Ghosts were prepared in 10 mOsm MTT buffer, pH 7.4, in the presence of varying concentrations of Mg as indicated. The ghost suspensions were assayed for (●) Hemoglobin,(▲) LDH, (■) Aldolase, in the presence of 0.05 vol.% TX-100

Fig. 21. The effect of Ca concentration in the washing buffer upon retention of hemoglobin, LDH and aldolase, by the erythrocyte ghosts



Ghosts were prepared in 10 mOsm MTT buffer, pH 7.4, in the presence of varying concentrations of Ca as indicated. The ghost suspensions were assayed for (o) Hemoglobin, (△) LDH, (□) Aldolase, in the presence of 0.05 vol.% TX-100

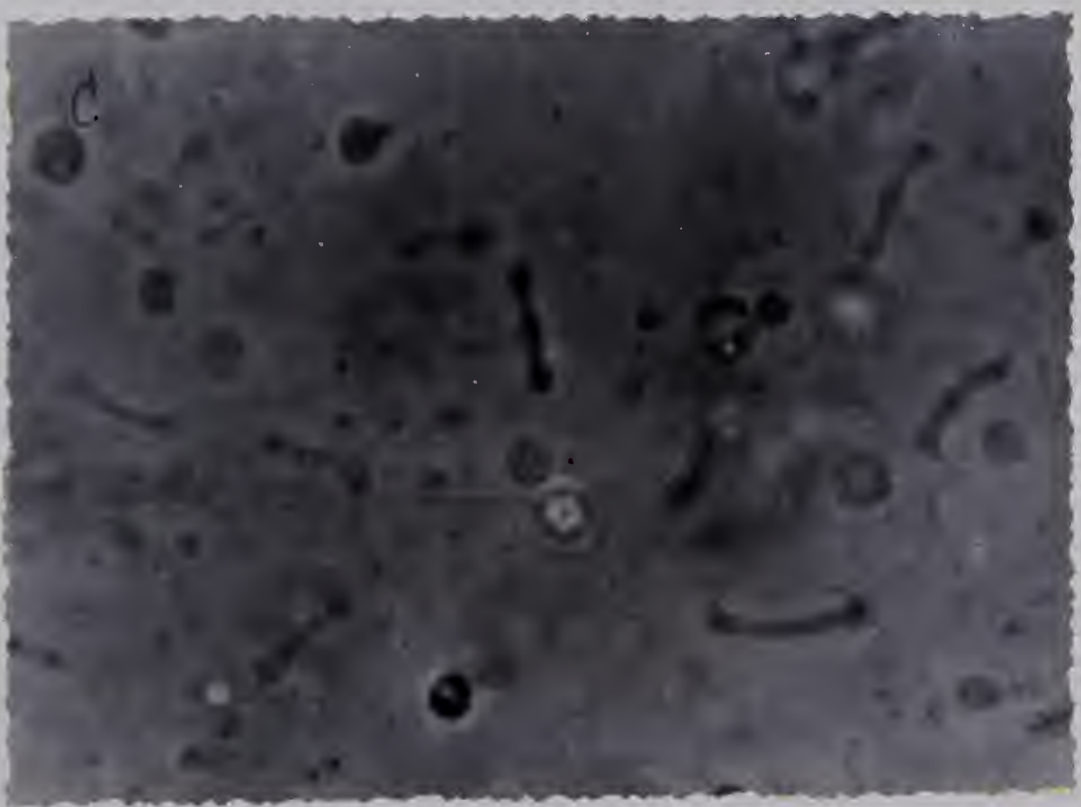
Ca concentration in the washing buffer upon the retention of hemoglobin, LDH, and aldolase activities by the erythrocyte ghosts. It is apparent on examination of these figures that, in the concentration range employed, neither Mg nor Ca has any significant effect upon the retention of aldolase by the erythrocyte ghosts. On the other hand, the concentration of Mg and Ca in the washing buffer has a profound effect upon retention of hemoglobin and LDH by the membranes. Of these ions, Ca proved to be more effective than Mg. As low as 5 μM Ca in the washing buffer increased the hemoglobin concentration of the ghosts above six-fold, and LDH activity nearly thirty-fold. Increases of the same magnitude due to the presence of Mg in the washing buffer were obtained at 400 μM Mg concentration.

The ghost preparations of Figs. 20 and 21 subsequently underwent microscopic examination. It was found that ghosts prepared in the presence of 400 μM and 200 μM Mg or Ca were intact, closely resembling those presented in Plate III-C and IV-A. At 100 μM concentration of the ions, although the large majority of the ghosts were intact, the presence of some fragments became apparent. As the concentration of the ions in the washing buffer was decreased, the number of intact ghosts declined, until at 1 μM concentration of the ions they became quite scarce in the preparations. At the same time the presence of an increasing number of ghost fragments and "vesicles" became more and more apparent. Vesicles were formed through division of the intact erythrocyte ghost. It was possible to view under the microscope the breakdown of apparently intact erythrocyte ghosts into either long ribbon-like tubules or vesicles.

Plate V. Ghosts were prepared in 10 mOsm MTT buffer, pH 7.4, in the presence of 50 μM Ca. The photomicrographs show the breakdown of ghosts into vesicles or tubules.


A


B


C

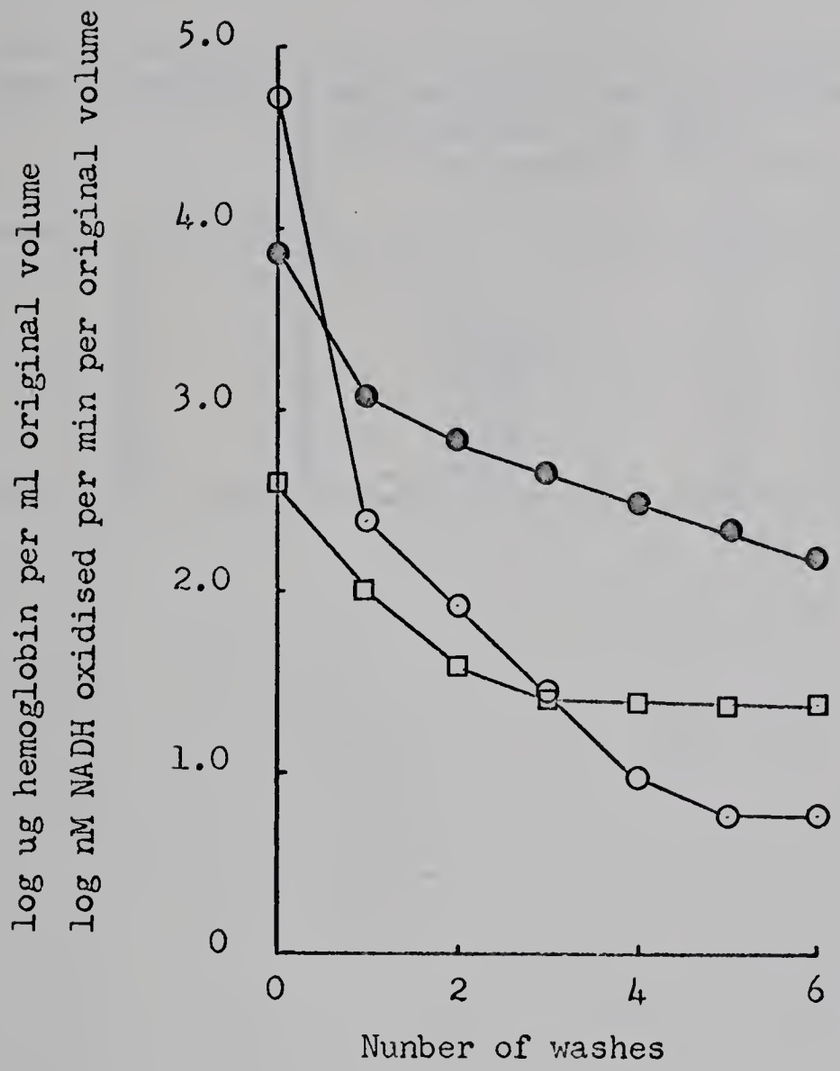
A set of photomicrographs taken of the ghosts prepared in the presence of 50 μM Ca is an attempt to illustrate this phenomenon in Plate V. Ghosts initially having a spherical shape became oblong, followed by a dumbbell shape, which divided into smaller vesicles. Alternately the dumbbell-shaped figures became more elongated, eventually forming long filamentous tubules with bulbar appendages at one end, or resembling beads on a string. Similar degradation could be observed at the lower concentrations of Mg-ghosts.

It is a well-known fact that the number of washings during preparation influences to some degree the final composition of the erythrocyte ghosts. Fig. 22 shows how this procedure affects the retention of hemoglobin, aldolase, and LDH by the erythrocyte ghosts. It may be seen that the greatest proportion of loss (four orders of magnitude) occurred in hemoglobin during the washing procedure, which was - for all practical purposes - removed completely from the ghosts by the fifth washing. The loss of the two enzymes from the ghosts was more moderate than that of hemoglobin. One notable difference in retention pattern of these enzymes is the fact that, while LDH was being continuously lost down to and including the very last wash, no aldolase activity was lost after the third washing. Table IX indicates that these losses were recovered in the supernatant liquid.

Kinetic and related studies

During the course of this investigation the question has arisen, whether the enzymes retained by the erythrocyte membrane are the same

Fig. 22. Dependence of hemoglobin and enzyme retention on the number of washes of the erythrocyte ghosts

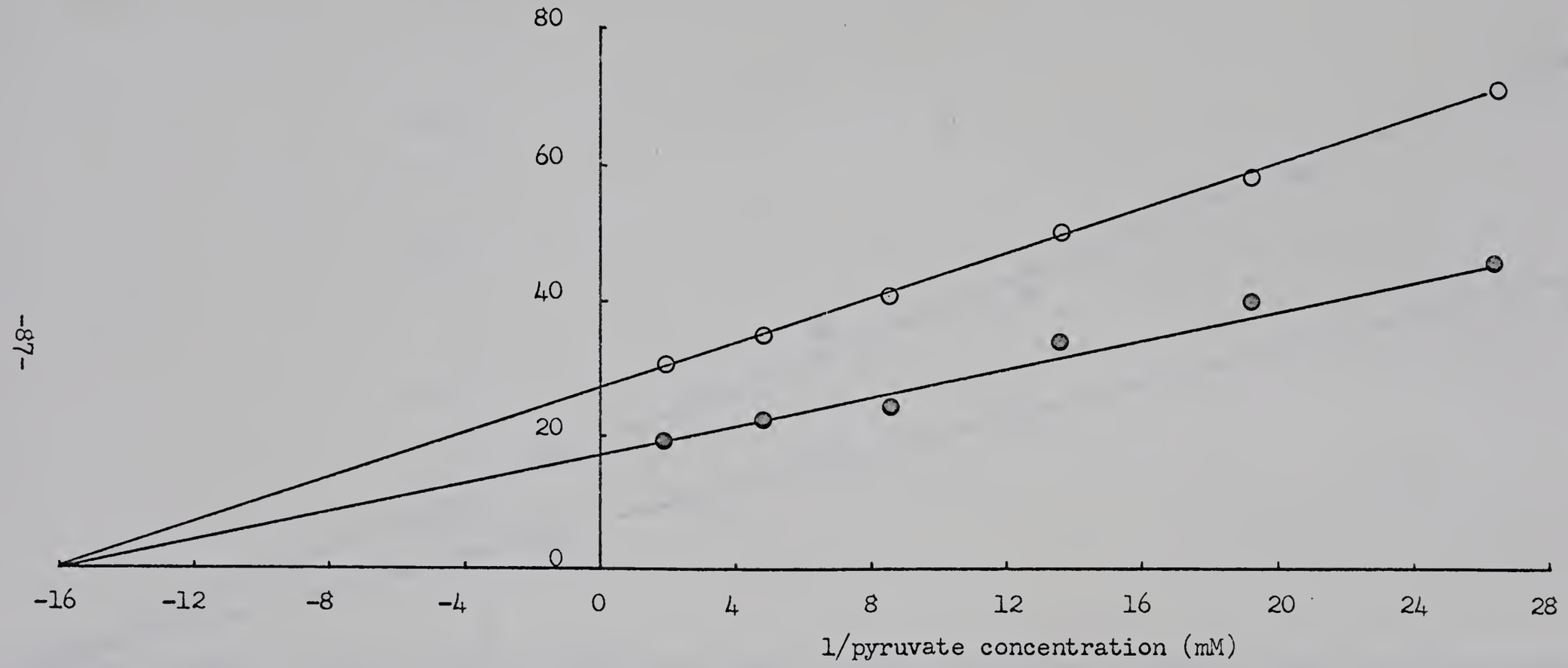


Ghosts were prepared in Tris buffered NaCl solution, 30 mOsm, pH 7.4. After every washing they were assayes for (o) Hemoglobin, (•) LDH, (□) Aldolase. The reaction mixture for enzymic assayes contained 0.03 vol.% TX-100

Table IX. Liberation of hemoglobin, aldolase and LDH into the supernatant liquid during the washing procedure of erythrocyte ghosts

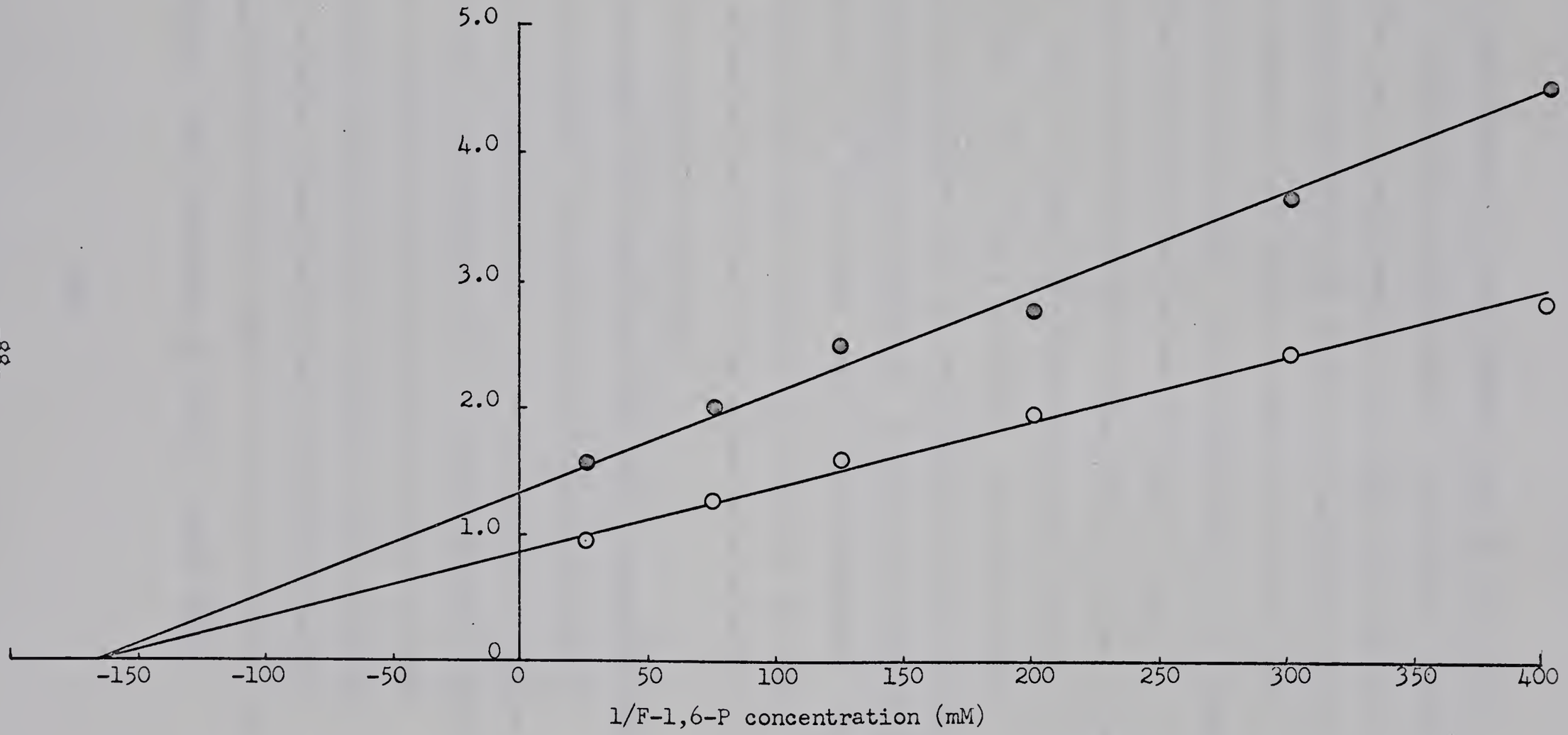
No. of washing	mg hemoglobin per ml original vol. of RBC suspension	IU per ml original vol. of RBC suspension	
		LDH	Aldolase
1	52	5.8	0.36
2	0.160	0.40	0.031
3	0.056	0.20	0.0130
4	0.019	0.170	0.0030
5	0.00	0.088	0.00
6	0.00	0.060	0.00

Fig. 23. Lineweaver - Burk plots for LDH of stroma-free hemolysate and of erythrocyte ghost



Ghosts were prepared in 30 mOsm Tris buffer, pH 7.4, and were compared for kinetics with stroma-free hemolysate. (o) Stoma-free hemolysate, (●) Ghost

Fig. 24. Lineweaver-Burk plots for aldolase of stroma-free hemolysate and of erythrocyte ghosts



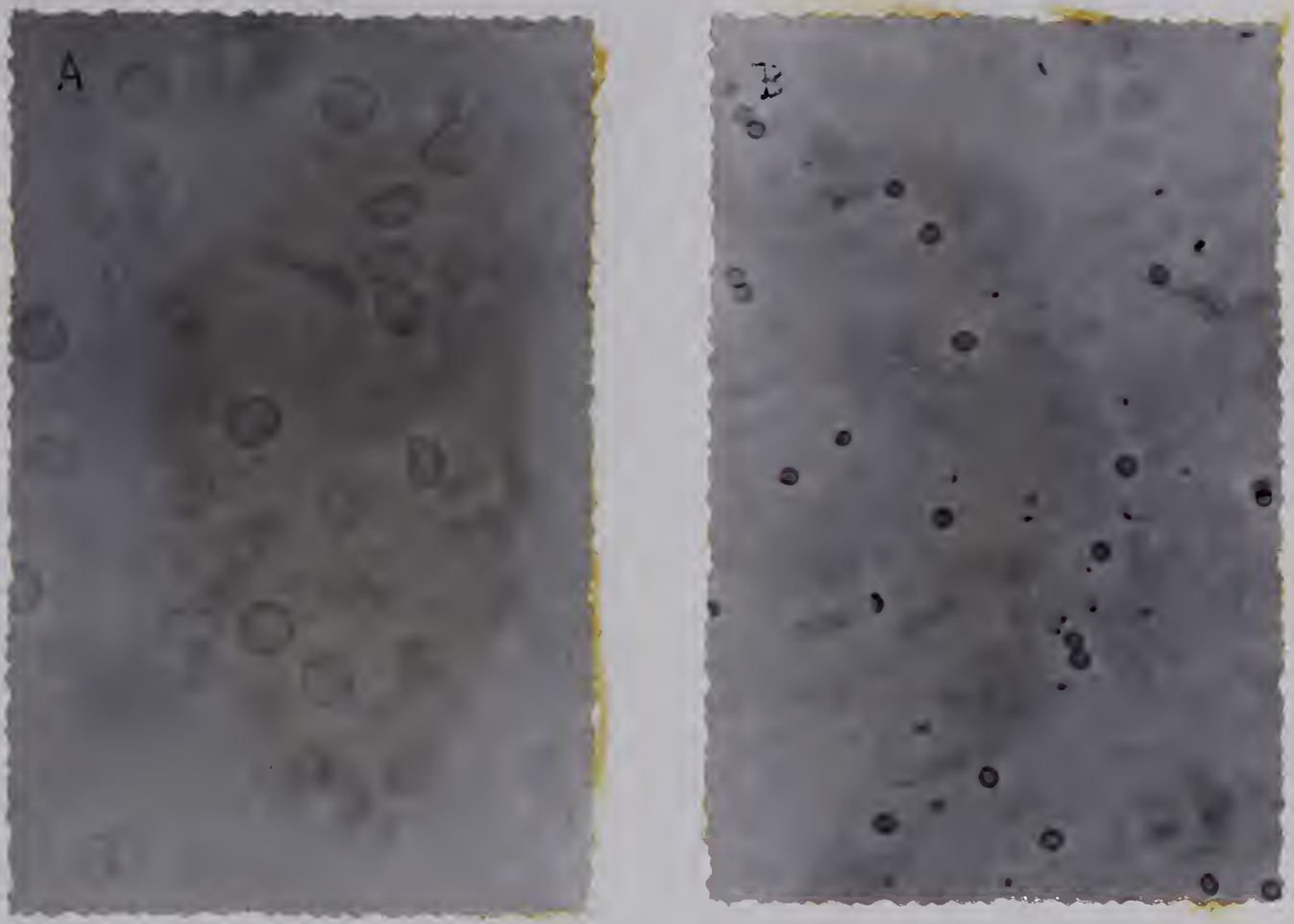
Ghosts were prepared in 30 mOsm Tris buffer, pH 7.4 and were compared for kinetics with stroma-free hemolysate. (o) Hemolysate, (●) Ghost

entities as those enzymes that are liberated from the cell during hemolysis, or whether they are iso-enzymes of each other. It was felt that some simple kinetic studies might answer this question.

Membrane-free hemolysate was obtained by hemolyzing a washed erythrocyte suspension in 30 mOsm Tris buffer, pH 7.4, which was centrifuged in the usual manner. After sedimentation of the ghosts, the stroma-free supernatant was removed and saved for kinetic studies, while the ghosts were futher washed four more times in the same buffer. Prior to assay, the ghosts were diluted six-fold in 0.05 vol.% TX-100. Fig. 23 is a Lineweaver - Burk plot obtained for a loosely-bound enzyme, LDH, of the erythrocyte ghost, as well as for LDH of the membrane-free hemolysate. It may be seen that the K_m is the same (6.4×10^{-5}M) for both. Fig. 24 is a Lineweaver - Burk plot of a firmly-bound enzyme aldolase, which also has the same K_m regardless of whether the source of the enzyme is the erythrocyte membrane or the stroma-free hemolysate.

The next enzyme to be studied was PGK, which as described under "Materials and Methods" was assayed in the backward reaction, by coupling with NADH-dependent TPDH. Its two substrates, 3-phosphoglycerate and ATP, are converted into 1,3-diphosphoglycerate and ADP. Kinetic data, obtained by using stroma-free hemolysate as enzyme were reproducible; however the data obtained by using erythrocyte ghosts were variable. Investigation into the possible source of error revealed that when ATP was added to the reaction mixture, the ghost suspension - made transparent by TX-100 - became opaque.

Plate VI. Ghosts were prepared in 30 mOsm Tris buffer, pH 7.4. (A) Control ghost suspension; (B) Mini ghosts; (C) Ghosts in the presence of 3 mM ATP.


A
B
C

This 'precipitation' of ghosts resulted in an increase of the absorbance at 340 nm, which fact could have accounted for the erratic behavior of the ghost PGK.

To investigate the 'precipitation' of the TX-100 treated erythrocyte ghosts by ATP, red cell ghosts were prepared in 30 mOsm Tris buffer, pH 7.4. An aliquot of the ghosts was treated with TX-100, 0.5 vol.%, to which ATP, 3 mM was added. Phase-contrast microscopic observation revealed that the ghosts had become dense, highly contracted spherical bodies as the result of this treatment. Plates VI -A & B are photomicrograms, contrasting the appearance of control and contracted "mini-ghosts", while Plate VI- C shows ghosts to which ATP was added. TX-100 treated ghosts could not be photographed, due to their extremely low contrast. It was actually possible to view under the microscope the process of contraction, by focusing on TX-100 treated ghosts, and injecting ATP solution under the edge of the cover glass.

Although microscopic studies were useful in observing shape variations of ghosts, such a method is not suitable for quantitative and kinetic analysis of volume changes. It was demonstrated by Teorell (9) and Wins & Schoffeniels (78) that an increase in apparent absorbance of a ghost suspension at 720 and 500 nm corresponds to a shrinkage of the ghosts, while a decrease corresponds to a swelling. Absorbance changes at 500 nm were used therefore to study the effect of TX-100 and ATP upon the ghost suspension as a function of time. Fig. 25 depicts such a recording. It may be seen that the absorbance of the ghost suspension remains stable, close to zero, when only TX-100

Fig. 25. The effect of ATP upon the absorbance of TX-100 treated ghosts

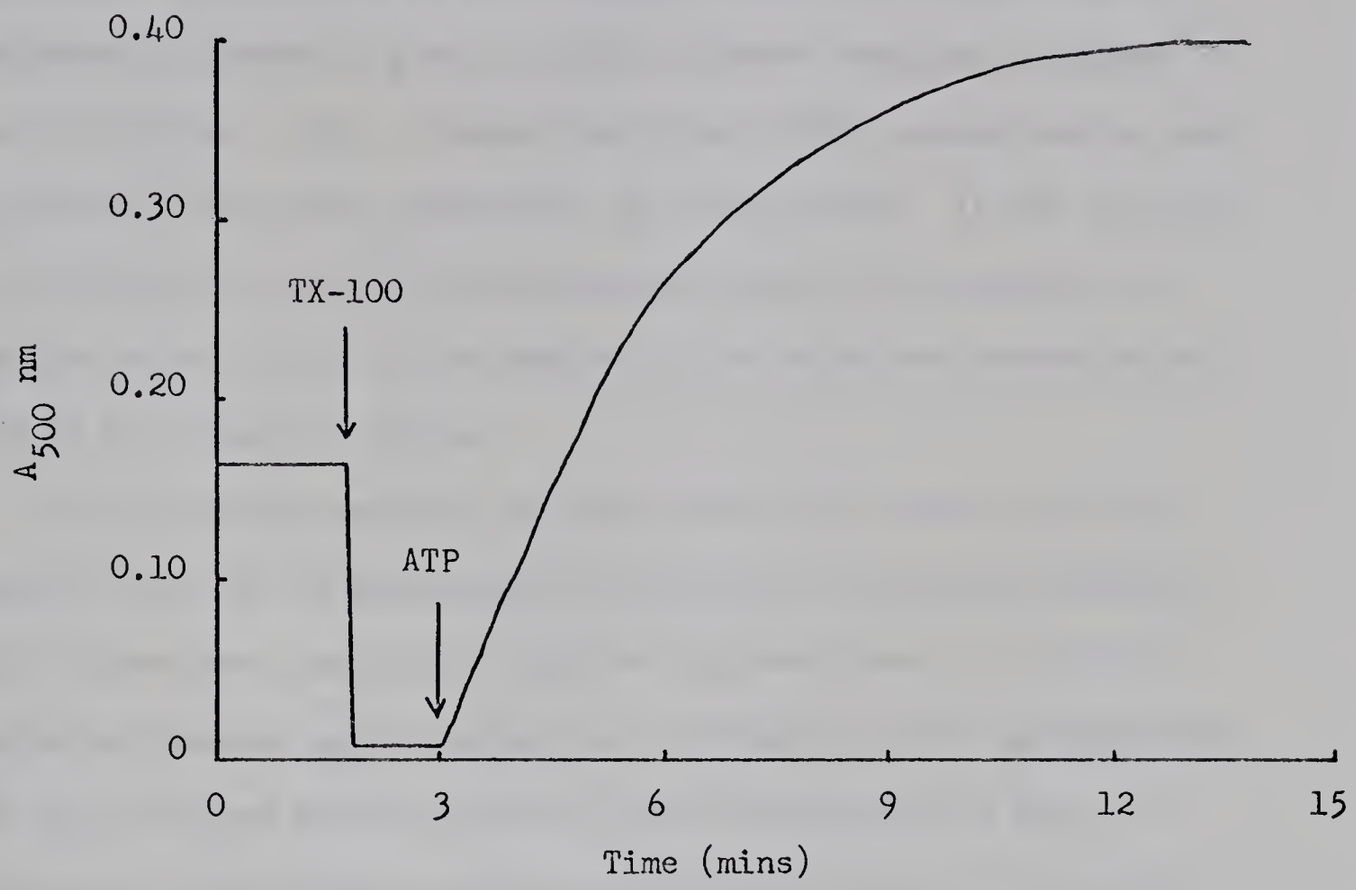
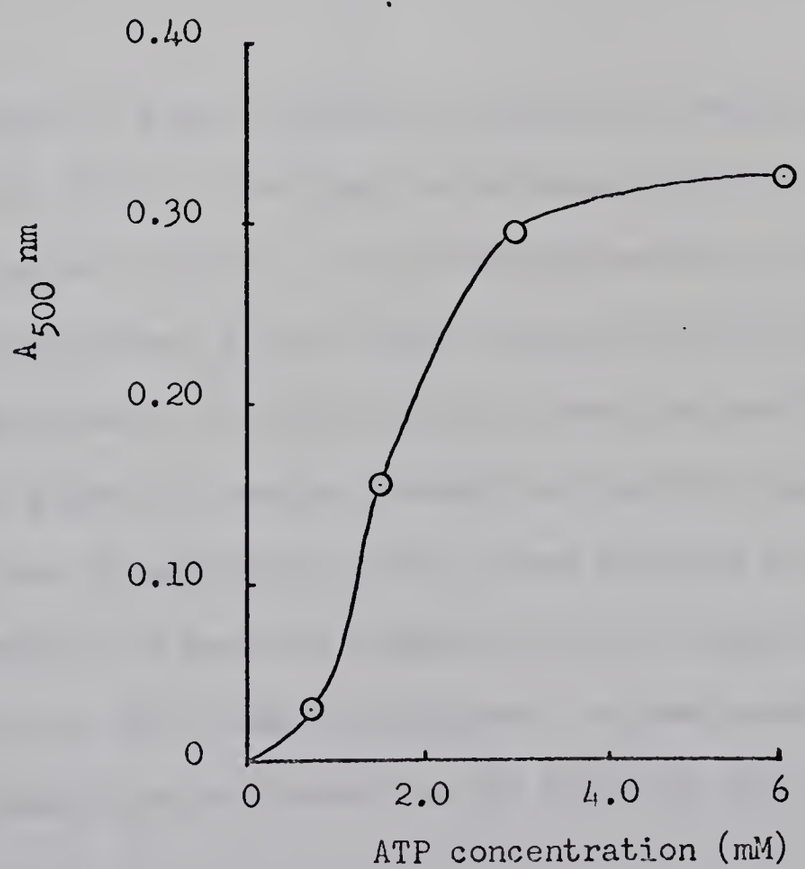


Fig. 26. The effect of ATP concentration upon the absorbance of TX-100 treated erythrocyte ghosts

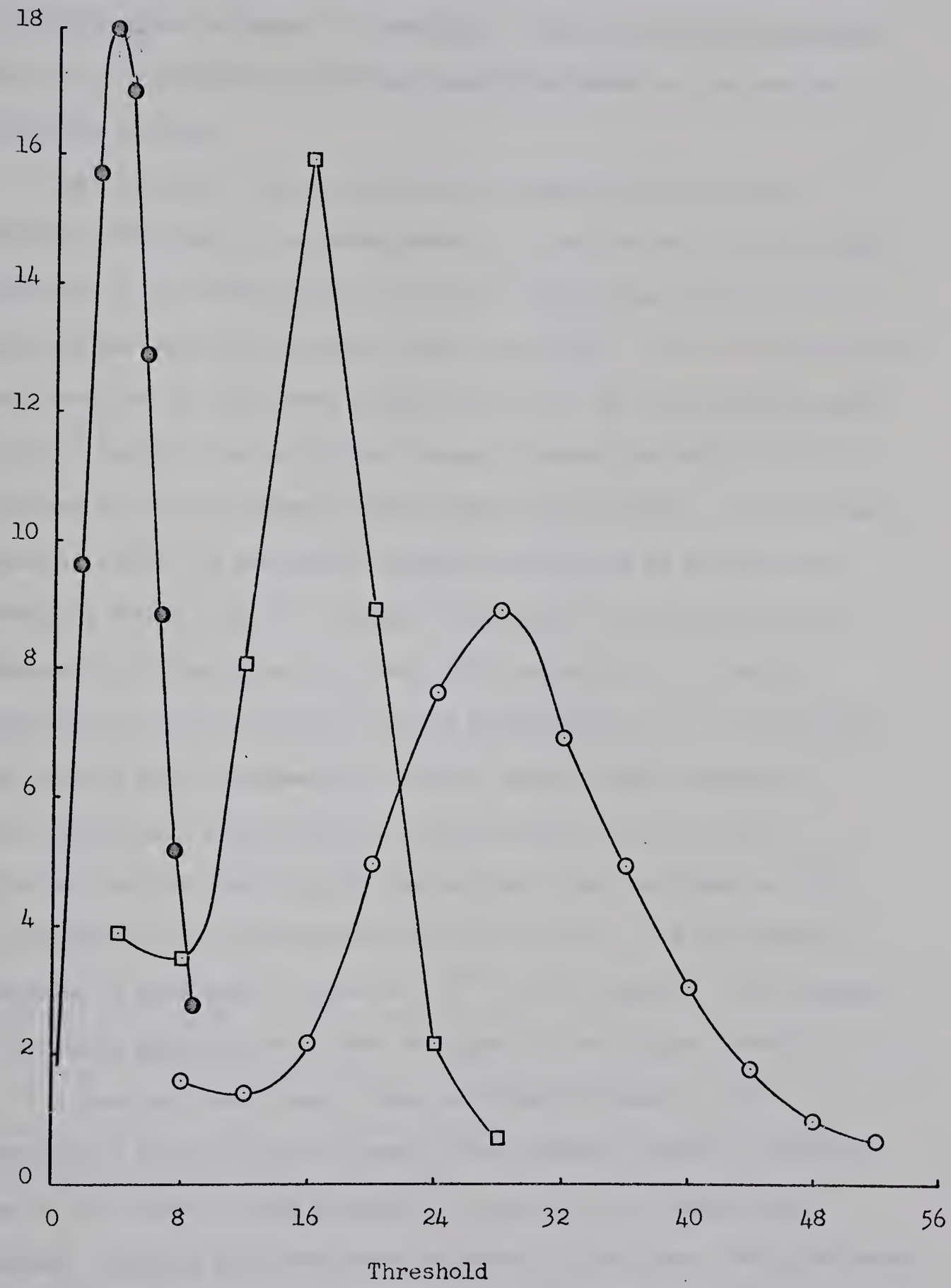


is present. On addition of ATP, however, there is a rapid rise in absorbance, followed by a more gradual increase reaching a plateau in about 10 minutes. Fig. 26 shows the effect of ATP concentration upon absorbance of the ghost suspension, at the plateaus. It may be noted that while Fig. 25 resembles the progress curve of an enzyme, the sigmoidal curve of Fig. 26 is similar to the substrate concentration curve of an allosteric enzyme.

In the previous section, Mg and Ca ions were shown to play an important role in the maintenance of erythrocyte membrane-integrity; their effects were therefore tested on the absorbance of a TX-100 treated erythrocyte ghost suspension. Although at 1 mM concentration both ions produced curves similar to that illustrated in Fig. 25, microscopic observations revealed that no contraction of the ghosts occurred comparable to that with ATP. The extremely faint ghosts due to the TX-100 treatment became, however, well outlined under the microscope.

Measurements of ghost volumes are possible through the use of the Coulter counter (76,77). The basic principles involved in these measurements are as follows. In a conducting medium such as 0.9% NaCl solution, direct current flows between two electrodes through a constricting aperture. A relatively non-conducting particle, such as an erythrocyte ghost, in passing through the aperture replaces an equivalent volume of suspending medium, thus produces a voltage rise by increasing the aperture impedance. The height of the pulse is proportional to the volume of the ghost. Pulses below and above any selected height can be blocked out by the lower and upper

Fig. 27. Size distribution curves of erythrocyte ghosts



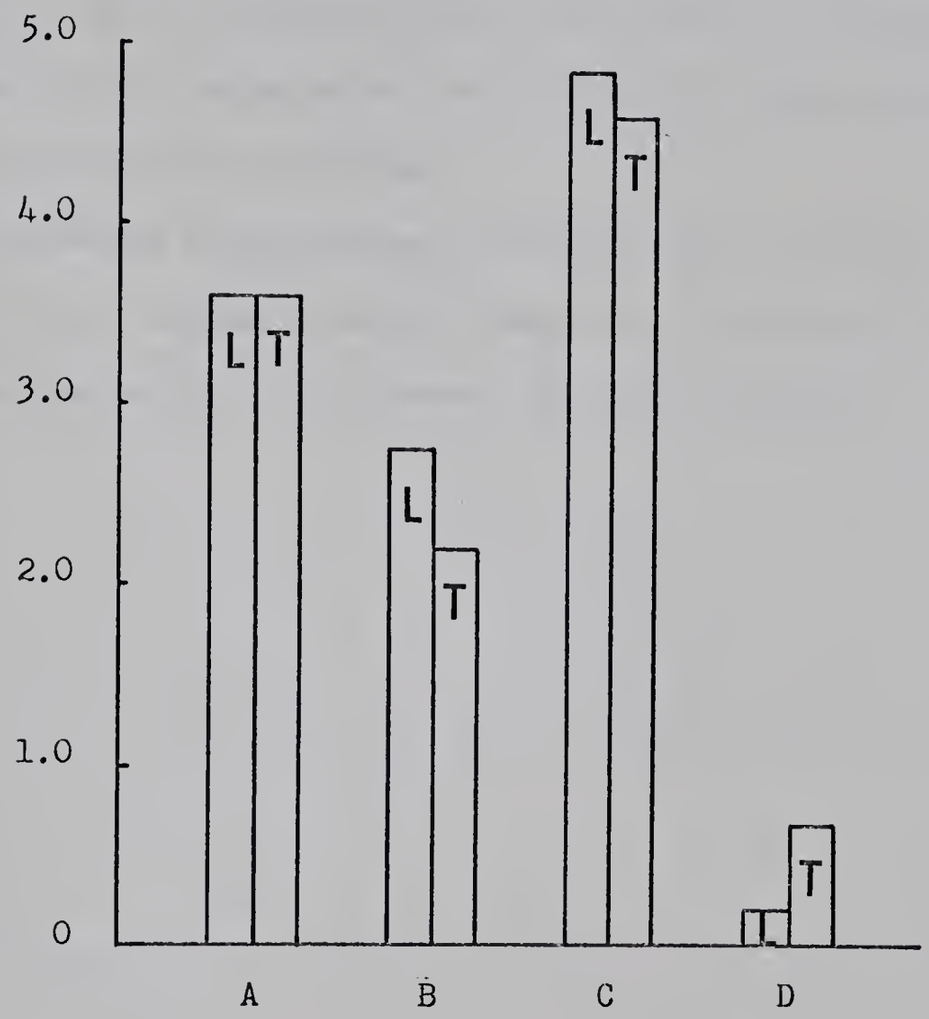
Ghosts were prepared in 30 mOsm Tris buffer, pH 7.4 and size distribution curves were obtained in 0.5% NaCl solution. (o) Control ghost suspension, (□) 3 mM ATP added to the stock ghost suspension, (●) TX-100, 0.5 vol.% followed by ATP, 3 mM, was added to the stock ghost suspension.

thresholds. Thus, counting of certain pulse heights, corresponding to certain ghost volumes, is possible. A particle-size distribution curve may be obtained, by taking successive counts at increasing threshold settings.

Fig. 27 depicts such particle-size distribution curves of control, ATP-treated, and mini-ghosts. It may be seen that the peak threshold of 28 obtained for the control ghosts was reduced to 16, when ATP was added to the stock ghost suspension. This corresponds to the reduction in peak ghost volume from 84 μ^3 of the control ghosts to 48 μ^3 for ATP-treated ghosts, using a conversion factor of 3.0 obtained for the instrument at the particular settings. An even more dramatic change in peak ghost volume was obtained on treatment of ghosts by TX-100 and ATP. A peak threshold of 4 obtained for this preparation corresponds to a peak ghost volume of 12 μ^3, which represent a volume reduction in the mini-ghost to $1/7^{th}$ of that of the control ghost suspension. Similar results were obtained by micro-hematocrit measurements. The hematocrit volume of 58% - obtained for the control ghost suspension - was decreased to 7.3%, on treatment of the ghosts with TX-100 and ATP. This represents a decrease in main ghost volume to $1/8^{th}$ of the original, which figure is in close agreement with that obtained by the Coulter counter.

It was felt that these volume and shape changes of the erythrocyte ghosts might influence their apparent enzymic activities. One of the loosely-bound enzymes, LDH, and the two firmly-bound enzymes, aldolase and TPDH, were selected for the test. While aldolase showed no significant variation in activity, LDH and TPDH activities

Fig. 28. The effect of ATP and Triton X-100 on the LDH and TPDH activities of erythrocyte ghost



Ghosts were prepared in Tris buffer, pH 7.4, 30 mOsm. They were assayed for (L) LDH, (T) TPDH activities:(A) in the suspending buffer; (B) in the presence of ATP, 3 mM; (C) in the presence of 0.5 vol.% of TX-100; (D) TX-100 treatment followed by ATP

were affected to some degree by the presence of TX-100 and/or ATP. As may be seen in Fig. 28 the most significant changes in enzymic activities were obtained in the mini-ghosts, where LDH activity was decreased to $1/24^{th}$ of the activity obtained in TX-100 alone, while TPDH activity was decreased to $1/7^{th}$. It was found that in the mini-ghosts 35-40% of the total ghost protein and 40-45% of cholesterol was liberated into the supernatant, but none of the above mentioned enzyme activities could be detected.

Other nucleotide triphosphates, CTP, GTP, UTP, as well as adenosine mono- and diphosphates were found to be ineffective in producing contraction in TX-100 treated erythrocyte ghosts.

IV. GENERAL DISCUSSION

In this investigation, the influence of osmolarity and pH of the wash medium, and the influence of Mg and Ca ions were studied upon retention of several enzymes by the erythrocyte ghost. To explain some of the results, phase-contrast microscopy was employed and found to be valuable. It has been clearly shown that no single set of conditions is sufficient to define the final composition of the erythrocyte ghost, but rather it is intricately influenced by several factors, to which the different enzymes do not react uniformly. It has been shown that the enzymes under study could be divided up into two groups, designated as loosely-bound and firmly-bound enzymes. The enzymes within one group responded similariy to each other under the different sets of conditions, but the responses of the two groups were different from each other. The enzymes in the loosely-bound groups are TPI; PK; LDH; GSH-P; and GSSG-R, while aldolase and TPDH form the firmly-bound group of enzymes.

The retention of enzymes by the erythrocyte ghost under different experimental conditions is especially relevent to discussion of the cell membrane. The preceding data serve as partial proof, that loosely-bound enzymes are cytoplasmic contaminents, either trapped by the semipermeable membrane of the ghost, or bound to the membrane by readily disruptable electrostatic bonds, and have no part in the fundamental membrane structure. Firmly-bound enzymes on the other hand seem to have a close association with the erythrocyte ghost, suggesting that these enzymes may indeed play an integral role in the ultrastructure of the erythrocyte membrane. It is felt that the

discussion of the results might become clearer in the light of the foregoing conclusion.

It has been shown in Figs. 4 - 7 that the osmolarity of the wash medium greatly influences the final composition of the erythrocyte ghost. The removal of the loosely-bound enzymes from the erythrocyte ghost was maximal between 5 and 10 mOsm buffer concentrations. At these low buffer concentrations ghosts were found to be totally fragmented (Plates III-A & B), allowing the soluble cytoplasmic components to escape from the particulate membrane fraction of the erythrocyte. An increase in the osmolarity of the wash medium is paralleled by the presence of an increasing number of intact ghosts, that is, an increasing number of semi-permeable membranes in the preparation. Consequently the escape of cytoplasmic constituents is hindered, resulting in an increased retention of these materials by the erythrocyte ghost. The intact erythrocyte ghost not only confines macromolecules - such as enzymes - within the interior of the membrane, but also hinders the movements of smaller molecules across it. This is quite apparent from the two activity profile curves. While the cryptic activity profile indicates the retention of the particular enzyme by the erythrocyte ghost at the different buffer concentration, the basic activity - profile curve represents the state of the permeability barrier. The gap between the two curves broadens as the osmolarity of wash medium increases, which reflects that the movement of substrate across the membrane is becoming more and more difficult as the ghosts retain their semi-permeable properties. A second conclusion which must be drawn from

the inspection of the basic activity profile curves - particularly those of LDH and PK (Figs. 4-A & 5-A) - is that, the critical point in the stretching of the membrane is at 30 mOsm buffer concentration. Above this point the activity profile curve declines, indicating that the permeability barrier is becoming more efficient. At lower than 30 mOsm buffer concentration, fragmentation of the membrane is beginning to take place, which is paralleled once again by the declining basic activity profile curve. This declining portion of the curve, however, represents decreased enzyme retention by the ghosts, as indicated by the cryptic activity profile curve.

The firmly-bound enzymes of the erythrocyte ghost have shown a profound difference from the loosely-bound enzymes, in the characteristics of their activity profile curves. The peak basic activity of these enzymes was obtained when ghosts were prepared in deionized water, but as the osmolarity of the wash buffer was increased, the activity profile curve declined. Although a decrease in ionic strength caused a significant enhancement of enzyme activity, the cryptic activity has never exceeded the activity of ghosts which were prepared in deionized water. Treatment of the ghost suspension with TX-100 caused a further enhancement of activity over the cryptic activity (Figs. 8-A & 9), in each and every preparation.

These results could be interpreted on the basis of a membrane model, which would incorporate protein in its ultrastructure in a more integrated role than simple physical adsorbtion of protein lining a bimolecular layer of lipids. The firmly-bound enzymes could then play an integral part in the basic membrane structure, facing with their

active site towards the interior of the membrane. When ghosts are prepared under conditions which result in production of membrane fragments, a certain portion of the interior membrane structure including the catalytic sites of enzymes is freed from the interior and brought in contact with the bulk of the liquid, thus becoming easily available to their substrates. With increasing osmolarity of the wash medium, an increasing proportion of structural enzymes would remain in the native state, which shows up as a continuous decrease in the activity profile curve. Mitchell et al. (47) obtained results for aldolase and TPDH similar to the basic activity profile curves indicated in Fig. 7. Consequently they concluded that since these enzymes are easily removed from the erythrocyte ghost by adjusting the ionic strength of the wash medium, they have no part in the fundamental membrane structure. These investigators however failed to recognize the cryptic nature of these phenomena. The evidence they used to disqualify these enzymes from a structural role in the membrane does in fact indicate a strong association of these enzymes with the erythrocyte membrane.

The data obtained by studying the effect of pH upon enzyme retention by erythrocyte ghosts complemented the results of osmotic studies. While changes in pH of the wash medium had only a slight influence on the retention of firmly-bound enzymes, they affected markedly the retention of hemoglobin and loosely-bound enzymes. The pH studies were carried out under conditions whereby the majority of the ghosts were either intact (20 mOsm preparation) or fragmented (5 mOsm preparation), in order to distinguish whether pH affects the

permeability barrier, or the binding of enzymes to the erythrocyte membrane. It was shown in Figs. 10 - 13, that the general patterns of activity for any particular enzyme are similar to each other, regardless of whether preparation of the ghosts was carried out in 5 or 10 mOsm buffer. This would indicate that the pH of the wash medium affects the binding of enzymes to the membrane, rather than the state of the permeability barrier. Had the latter been true, the activity of the 5 mOsm preparation would have been expected to be uniform throughout the pH range under study.

Reports in the literature (14,47,68) indicate removal of non-hemoglobin proteins from the erythrocyte ghost under the influence of pH; attempts to characterize these proteins however were unsuccessful (47). Although these ghosts are structurally intact (47), it is claimed that the use of a higher pH leads to the partial disintegration of the erythrocyte membrane (68). The data presented in Figs. 12 & 13 clearly indicate that the loosely-bound group of enzymes are at least partially responsible for the loss of non-hemoglobin proteins above pH 7.0. One may put this in another way and say: loosely-bound enzymes along with hemoglobin specifically adhere to the erythrocyte membrane at pH's lower than 7.0; one could speak of adsorption of proteins to the membrane below this pH. Considering that the physiological pH of the red cell is 7.4, it would not only sound more acceptable, but also, it would put the whole question into a different perspective. The question whether structural protein is lost from the membrane above pH 7.0, would become whether cytoplasmic components are contaminating the membrane below this pH. If the question is

acceptable in this form, experimental data indicate that hemoglobin (14) (see also Fig. 10) and the loosely-bound enzymes (Figs. 12, 13, and Tables VI & VII) are indeed specifically adsorbed to the membrane below pH 7.0, while the firmly-bound enzymes (suspected structural membrane components) are not affected. If however the question is not acceptable in the above form, the data would indicate that the loosely-bound enzymes along with hemoglobin are held to the membrane by readily disruptable electrostatic bonds, suggesting no part in the fundamental membrane structure. Firmly-bound enzymes on the other hand are not so affected by changes in pH, implying that these particular enzymes are specifically and tightly bound in the membrane itself, suggesting a structural role within the membrane architecture.

Data presented in Tables VI & VII indicate that the removal of loosely-bound enzymes from the erythrocyte ghost does not mean an irreversible structural change of the membrane. Once the ghost preparation is carried out, these enzymes can still be removed from, or recombined with the membrane, through a small change in pH of the suspension. Firmly-bound enzymes on the other hand can neither be removed from nor recombined with the membrane under similar conditions.

In studying the role of Mg and Ca in relation to the red cell membrane, it has been found that these ions greatly influence not only the retention of hemoglobin and loosely-bound enzymes by the erythrocyte ghost, but that they also play a profound role in the maintenance of membrane integrity.

When either one of these ions was incorporated into the wash medium, the ghosts retained substantially more hemoglobin and LDH than their counterpart prepared in the absence of these ions (Figs. 16 - 18). The increased enzyme retention by Mg- and Ca-ghosts would have gone unnoticed however, and the treatment of ghosts with TX-100 been omitted from these experiments. So much so, that on the basis of the 30 mOsm preparation (Fig. 18), one would have actually observed a decrease in enzyme retention by the Mg-and Ca-ghosts. Although the total hemoglobin content as well as the LDH activity was higher in the 30 mOsm ghost preparation, the influence of Mg and Ca ions on retention of these proteins was more evident at the lower ionic strength. This can partly be explained in the light of the results obtained by phase contrast microscopy, which indicated that the presence of Mg or Ca ions in the wash medium protects the erythrocyte ghosts against hypotonic disintegration.

It appears, however, that the action of Mg and Ca ions on the erythrocyte ghost is two-fold, in regard to retention of hemoglobin and LDH: A) By maintaining membrane integrity against hypotonic disintegration, they decrease the free escape of cytoplasmic constituents from the erythrocyte ghost. Through this action, the level of hemoglobin and LDH in the 10 mOsm Mg- and Ca-ghost preparations would have been brought up to the level of the 30 mOsm standard-ghost preparation, since this is the minimal osmotic strength still producing physically intact ghosts. B) In addition to their protective action, Mg and Ca ions seem to affect the permeability properties of the erythrocyte ghost. This is indicated by the

following: I) The retention of hemoglobin and LDH by the 10 mOsm Mg- and Ca-ghosts is significantly higher than the 30 mOsm standard-ghost preparation: II) Mg- and Ca-ghosts prepared in the 30 mOsm buffer have retained substantially more hemoglobin and LDH than the corresponding standard-ghosts; III) The latent activities of Mg- and Ca-ghosts in the 30 mOsm preparations are lower than the corresponding activity of standard-ghost preparation, indicating that in these ghosts, not only the escape of macromolecules is hindered, but also the movement of small-substrates molecules across the membrane has become more difficult; IV) Although a six-fold dilution of Mg- and Ca-ghost with deionized water resulted in an enhancement of LDH activity, it fell short of the activity obtained in the 30 mOsm standard-ghost preparation, not to mention the activity that was obtained on TX-100 treatment. The "stretching" of the membrane by hypotonic shock and its effect on membrane permeability was carried a step further, when the ghost preparations were diluted with varying volumes of deionized water (Fig. 19). The standard-ghost preparation rapidly reached a plateau in LDH activity, at about 6 mOsm buffer concentration, where the majority of the ghosts would be fragmented. A slight enhancement in activity due to the treatment with TX-100, could be attributed either to the presence of some intact ghosts (highly unlikely at the higher dilutions), or to the possibility that the vesicles formed during hypotonic disintegration, locking the enzyme inside, may have retained some of the semi-permeable properties of the membrane. Mg- and Ca-ghosts similarily underwent a rapid rise in LDH activity at the lower end of the dilution scale, followed by a more moderate but

continuous rise in activity, up to 20-fold dilution with deionized water. Even at this high level of dilution, the activity of Mg- and Ca-ghosts were 27% and 34% respectively, of the activity obtained on treatment with TX-100.

The retention of firmly-bound enzymes by the erythrocyte ghosts, under the influence of Mg or Ca ions, was radically different from the retention pattern of the loosely-bound enzymes (Table VIII). One of the differences between the two groups of enzymes is that, while the latent activity of the loosely-bound enzymes is higher in Mg- and Ca-ghosts than the activity of the standard-ghosts (Ca-ghosts being the highest), firmly-bound enzymes show decreasing activity in the presence of the ions, with Ca-ghosts giving the lowest activity. The second important difference between the two groups is that, while Mg and Ca ions greatly enhance the retention of loosely-bound enzymes, the retention of firmly-bound enzymes is completely unaffected by these ions.

The results obtained by studying the effect of Mg and Ca concentration in the wash medium upon hemoglobin and enzyme retention, as well as on the integrity of the erythrocyte ghost were complementary to the above. It was found that, in the concentration range employed, ghosts have gone through a spectrum of physical states; from being intact above 200 μM, to totally fragmented at the lowest Mg and Ca concentrations. Accordingly, the retention of hemoglobin and LDH increased with increasing Mg and Ca concentration, while the retention of aldolase was unaffected by changes in concentration of these ions (Figs. 20,21).

These results are in good agreement with the roles that have been assigned to these ions. By maintaining membrane integrity, Mg and Ca ions influence the retention of loosely-bound enzymes, but they are not expected to affect the retention of an enzyme which is part of the fundamental membrane structure. Their action on membrane permeability on the other hand, does hinder the movement of substrate molecules in reaching their buried enzymes, resulting in the decreasing latent activity. These results are perfectly harmonious with previous results obtained through other lines of investigation, and once again seem to emphasize the structural role that these firmly-bound enzymes may play in the ultrastructure of the erythrocyte membrane. Once again they seem to stress the point, that the loosely-bound enzymes along with hemoglobin are trapped cytoplasmic contaminants, that might transiently be associated with the membrane, but have no role in the fundamental membrane structure.

Nakao et al. (79) have demonstrated that the shape of the erythrocyte is dependent on its ATP content, and subsequently suggested the presence of an ATP-dependent contractile system in the erythrocyte. Whittam (80) has pointed out that the swelling of cells (observed by Nakao et al. when the ATP content of the erythrocyte dropped to 10% of normal), could be due to failure of active cation transport. Ohnisi (81) reported the isolation of an actomyosin-like protein from the erythrocyte, and the same is suggested by the studies of Wins and Schoffeniels (78) on red cell ghosts.

During the course of this investigation, it was found that erythrocyte ghost treated with TX-100 form "mini-ghosts" in the

presence of ATP. Since mini-ghosts appear as small dense spherical bodies under the microscope, the presence of a contractile protein in the membrane was highly suggestive. Consequently the phenomenon was investigated at some length.

Since Nakao et al. (79) observed shape variations microscopically, their method was not suitable for quantitative and kinetic analysis of volume changes. Wins and Schoffeniels (78) recorded absorbance changes at 500 nm photometrically as a function of time, correlating it with volume changes of the erythrocyte ghost. This method, "if at all" suitable for kinetic analysis of volume changes, is not adequate for a quantitative analysis. In this study, it was decided to correlate the two methods mentioned above, and in addition bring in some new techniques for the direct quantitation of volume changes.

A spectrophotometric recording reproduced in Fig. 25 depicts changes in absorbance of a ghost suspension, upon addition of TX-100 and ATP. The drop in absorbance upon addition of TX-100 is supposed to indicate swelling of the ghost suspension, according to the theory presented earlier (9,78). Although good evidence is not available to negate this, microscopic observations seem to suggest that no swelling of ghosts occurred on TX-100 treatment. It is felt that the ghosts were made more transparent by partial solubilization, through the action of TX-100 on the lipoprotein complexes of the erythrocyte membrane. On the other hand, the increasing absorbance of the ghost suspension, upon addition of ATP, indeed reflects a shrinkage of the ghosts, as it was shown by other lines of evidences. In addition to phase-contrast microscopy (Plate VII), the formation of

mini-ghost was demonstrated by particle size distribution in the Coulter counter (Fig. 27), as well as by measurements of hematocrit volumes. These measurements indicated a contraction of mini-ghosts to $1/7^{th}$ to $1/8^{th}$ the volume of control ghosts. Very similar curves to that of Fig. 25 can be obtained, by addition of 1 mM of either Mg or Ca, to TX-100 treated erythrocyte ghosts. Phase microscopic study of these suspensions indicated, however, that no contraction of the ghosts had taken place, but that they had regained their original appearance. Contraction of the erythrocyte membranes into mini-ghosts was found to be specific for ATP, as neither ADP or AMP nor the other nucleotide triphosphates could be used as a substitute.

The results of this investigation can be best explained on the basis of a membrane model, which would incorporate protein into its fundamental architecture. Such a model has been proposed (36 - 39) and it was described in the "Introduction" (Diagrams 4 & 5). The model emphasizes the lipoprotein nature of the membrane and its division into protein-coated globules, as opposed to the Danielli - Davson theory of a continuous lipid bilayer to which protein is ionically bound. According to Green and Perdue (38,39), membranes are made up by different repeating units; all repeating units are constituted of a fixed sector or basepiece, which is invariable and indispensable to membrane formation and continuity. To the basepieces may be attached a detachable sector or headpiece, which is not necessary to continuity of the membrane, but is a projection therefrom.

A close association of firmly-bound enzymes with the erythrocyte ghosts was clearly shown throughout this investigation, subsequently their participation in the membranes basic architecture was proposed.

Loosely-bound enzymes on the other hand show only a weak association with the erythrocyte membrane. It was proposed that these enzymes are cytoplasmic contaminants, either trapped within the membrane confine by the permeability barrier or held to the membrane by weak, readily disruptable electrostatic bonds. The possibility for these enzymes to be associated with the erythrocyte membrane can not be excluded however. Since the headpieces are proposed to be readily detachable with no subsequent alteration to the membrane structure, it is conceivable that the loosely-bound enzymes are identical to these detachable sectors, but are indistinguishable from cytoplasmic contamination on the basis of this investigation. Such an admittedly speculative proposal actually was advanced by Baum et al. (81).

The models proposed by Korn, Lenard and Singer, Green and associates are deficient in recognizing the role which may be played by Mg and Ca ions, in the anatomy of membrane ultrastructure. A membrane model proposed by Whittam (82) incorporates Ca (not Mg) as a functional membrane component, but his model is incompatable with the above proposals in other regards. As a result of this investigation it is felt that even the most schematic treatment of the subject should include some sort of reference to these ions.

In the course of this investigation it was shown that ghosts form either long ribbon-like tubules or vesicles when the ionic strength of the suspending medium is reduced. This instability of the membrane at low ionic strength would suggest that the interaction between the repeating units is not entirely hydrophobic, but rather, at certain regions it may be ionic. This is also indicated though the protective action of either Mg or Ca, against hypotonic disintegration of the erythrocyte ghost. These ions may act by bridging the repeating units of the membrane, in the negatively charged neighbouring regions. (A structural protein isolated from the erythrocyte ghost by Maddy (83) was shown to be acidic in nature). Although this type of mechanism may also account for the decreased permeability of the erythrocyte ghosts when they are prepared in the presence of these ions, or for the increased retention of hemoglobin and loosely-bound enzymes, a second alternative should be also considered here. Mg and Ca ions apart from bridging the negatively charged protein regions of the neighbouring basepieces may similarly attach or stabilize the projecting elements or headpieces to the membrane-forming sectors. Glycolytic enzymes and hemoglobin have been implicated in the formation of headpieces (81). Alternatively they could link cytoplasmic protein to the membrane continuum, creating a gel state, which could not only affect membrane permeability or retention of hemoglobin and loosely-bound enzymes, but which might also stabilize membrane integrity. The latter possibility is particularly favoured if one considers the effects of Mg or Ca upon TX-100 treated ghosts, which regain their original appearance upon addition of these ions to their suspension.

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